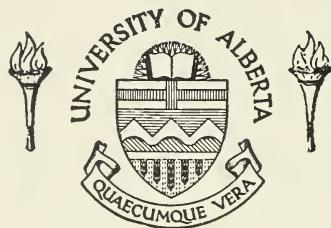




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THE UNIVERSITY OF ALBERTA

AN INVESTIGATION OF THE BACTERIA ASSOCIATED  
WITH THE HORN FLY, SIPHONA IRRITANS,  
AND THE METABOLIC BEHAVIOR  
OF THE INDIVIDUAL ORGANISMS

A DISSERTATION  
SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES  
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE  
OF MASTER OF SCIENCE

FACULTY OF MEDICINE  
DEPARTMENT OF BACTERIOLOGY

by  
George William Schwindt

EDMONTON, ALBERTA,  
SEPTEMBER, 1952.



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Thesis  
1952  
# 69

University of Alberta

Faculty of Medicine  
Department of Bacteriology

The undersigned hereby certify that they have  
read and recommend to the School of Graduate Studies for  
acceptance, a thesis entitled "An Investigation of the  
Bacteria Associated with the Horn Fly, Siphona irritans,  
and the Metabolic behavior of the Individual Organisms"  
submitted by George William Schwindt, B. Sc., in  
partial fulfilment of the requirements for the degree  
of Master of Science.

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## ABSTRACT

This work was undertaken as part of a larger project which comprised an investigation into the biology and behavior of the horn fly, Siphona irritans. The main project arose from the desirability of controlling the horn fly population on range cattle. One of the chief problems to be considered was the difficulty of rearing horn flies in captivity in order to study their biological activities more adequately. The purpose of this work was to investigate the bacteria associated with Siphona irritans in its natural environment and from this, determine the importance of these bacteria to the fertility of the horn fly.

The present thesis is a systematic account of micro-organisms and particularly bacteria associated with wild and laboratory reared horn flies. Organisms were isolated from the external surfaces of wild flies, from their crushed bodies after the external surfaces had been disinfected, and from their intestinal tracts after dissection. Limited studies were made of ova, larvae, and pupae of wild horn flies and of the skin and faeces of cattle. The conclusion drawn from these studies is that the bacterial flora of the horn fly is derived from its environment and directly related to it.

During this project several interesting observations were made. Although in general the intestinal bacteria of the horn fly appeared representative of its environment



yet they were obviously influenced by some unknown endogenous factor. Early season flies were found to contain mainly Gram positive cocci whereas in late season flies small Gram negative rods predominated. Although some degree of mutual antagonism was shown to exist in strains of intestinal bacteria the change in bacterial population cannot be ascribed to this. Nevertheless this antagonism is probably associated with the relative proportions of individual strains at any one time.

During this investigation it was found that the fertility of Siphona irritans is not determined by any factor directly derived from its intestinal or environmental bacteria. A study of the nitrogen metabolism of the bacteria derived from the intestine of the horn fly showed that certain strains were capable of synthesizing a number of amino acids. This raises a new hypothesis, that such bacteria, although apparently not related to a fertility factor, may yet play a vital role in the insect's metabolism.

Much work was done on the elaboration of techniques necessary to handling, rearing, sterilizing and dissecting horn flies and these methods have been described in detail. It is hoped that later workers who may wish to pursue further investigations in this practically unexplored field may be saved much time and trouble by consulting the descriptions.



### ACKNOWLEDGEMENT

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TABLE OF CONTENTS



## TABLE OF CONTENTS

	PAGE
<u>OUTLINE of MAIN PROJECT and SCOPE of PRESENT THESIS</u>	
Outline of Main Project and Scope of Present Thesis....	1
Some Entomological Considerations.....	2
Life History and Habits.....	3
<u>INTRODUCTION</u> .....	10
The Importance of Microorganisms in Animal Nutrition .	11
<u>MATERIALS and METHODS</u> .....	18
Collection, Handling, and Rearing of <i>S. irritans</i> .....	19
Isolation of microorganisms from the External surfaces of <i>S. irritans</i> .....	23
Selection of a suitable Disinfectant for the external surfaces of <i>S. irritans</i> .....	25
The use of Thioglycollate Medium to detect possible flaws in the Disinfectant procedure.....	28
Further problems in Disinfection of the External surfaces of <i>S. irritans</i> .....	31
Isolation of Microorganisms from the Interior of <i>S. irritans</i> .....	33
Dissection of <i>S. irritans</i> .....	34
A Method for the Rapid Removal of Intestinal Tract....	36
Preparation of Faecal Smears.....	38
Preparation of Histological Sections.....	39
Testing for Antagonistic action among organisms isolated from <i>S. irritans</i> .....	41



TABLE OF CONTENTS cont'd.

	PAGE
Isolation of Microorganisms from the Natural environment of <i>S. irritans</i> .....	48
Classification of Microorganisms.....	50
Some Tests used in the Identification of bacteria... Experiments in which Bacteria were fed to <i>S. irritans</i>	54 56
An Experiment in which flies were fed on a calf.....	59
A Study of the Amino Acid Metabolism of the Normal intestinal flora of <i>S. irritans</i> .....	61
 <u>MICROORGANISMS ISOLATED DURING the PRESENT PROJECT and their METABOLIC BEHAVIOR.....</u> 63	
Fungi, Yeasts, and Molds associated with <i>S. irritans</i>	64
Bacteria associated with <i>S. irritans</i> .....	66
Bacteria Isolated from the faeces of cattle.....	75
Bacteria Isolated from the skin of cattle.....	77
Bacteria Isolated from the external surfaces of wild <i>S. irritans</i> .....	79
Bacteria Isolated from the interior of wild <i>S. irritans</i> .....	81
Bacteria Isolated from the intestinal tract of wild <i>S. irritans</i> .....	83
Bacteria Isolated from the interior of ova, larvae, pupae, and unfed flies.....	86
Bacteria Isolated from laboratory reared flies fed <i>M. flavus</i> .....	89



TABLE OF CONTENTS cont'd.

	PAGE
Amino acid Metabolism of Normal Intestinal bacteria on Complex and Simple Media.....	90
<u>SUMMARY and CONCLUSIONS</u> .....	104
Summary.....	105
Conclusions.....	106
<u>LITERATURE CITED</u> .....	108
<u>APPENDIX</u> .....	115



OUTLINE OF MAIN PROJECT

and

SCOPE OF PRESENT THESIS



Outline of Main Project and Scope of Present Thesis.

This work was undertaken as part of a larger project which comprised an investigation into the biology and behavior of the horn fly, Siphona irritans.

As a cattle "pest" the horn fly has few if any equals; in parts of the United States (California) it is regarded as the most serious one encountered. The horn fly is of comparatively recent introduction into North America from Europe where it has long been recognized as an important cattle pest. According to the United States Bureau of Entomology it was first reported in 1887 in Camden, New Jersey; by 1892 it was found over the entire continent from Canada to Mexico and from the eastern sea-board to the Rocky Mountains. In Alberta the flies are found mostly in the southern half of the province where they appear early in the spring and become most abundant in late summer and autumn. Cattle are the principal hosts of the horn fly and the dark coloured breeds are said to be attacked more than are the lighter coloured breeds (Marlatt, 1910; Bruce, 1940). Observations made by McLintock (1951) in southern Alberta and Hammer (1941) in Denmark indicate that horn flies are more abundant on bulls, while on cows the short-haired breeds and individuals are preferred. Hammer (1941) concluded that temperature and colour played little part in the attraction of the host. Horses, mules, sheep, goats, dogs, and man are attacked occasionally.



(Howard and Marlatt, 1889; Marlatt, 1910; Bruce, 1940; Hammer, 1941). The damage caused by the horn fly is brought about chiefly through irritation and annoyance of cattle in pasture. This results in disturbed feeding and improper digestion, which in turn leads to loss of flesh in range cattle and reduction of milk in dairy cattle. The loss of blood must be considerable when thousands of these flies attack any one animal. Laake (1946) showed the extent to which flesh production was reduced by the horn fly among cattle in Kansas. Three herds of cattle were sprayed with 0.2% DDT suspension. For every pound of insecticide applied, these herds registered weight gains of 1202, 1285, and 2306 pounds more respectively than comparable untreated herds. Calves gained 96, cows 46, and mixed herds 30 pounds per head more than in control herds. In Ontario and Quebec the loss of meat and milk through horn fly infestation was estimated by Fletcher (1892) to be as high as fifty per cent. In southern Alberta, due to the apparent preference of flies for bulls, the ranchers claim that the attacks of this fly also reduce the calf crop.

#### Some Entomological Considerations

The horn fly derives its name from a practice which the fly sometimes has of clustering at the base of the horns of cattle. According to Howard and Marlatt (1889, 1889a) and Marlatt (1910) the horn-clustering habit is only noticed in the spring and early summer when the flies are very abundant and averaging more than 100 flies per animal. But in Haiti,



Myers (1938) observed the horn clustering practice only when the flies were least abundant (50 to 100 per animal) and usually in cool misty weather or on chilly shaded slopes. To the authors knowledge the horn-clustering practice has never been observed in western Canada. But when large numbers of flies are collected and placed in a flask considerable condensation is formed and the flies tend to cluster in a ball on the bottom of the flask. These observations suggest that clustering is a reaction to high humidity.

#### Life History and Habits

Little was known about the life history of the horn fly until Howard and Marlatt (1889, 1889a) and Smith (1889, 1890) published the results of their observations made in the eastern United States. These observations have been widely quoted and, together with the summary published by Marlatt (1910), have formed the basis of text book descriptions ever since. In a series of papers published between 1938 and 1948 Bruce contributed additional facts regarding the life history and habits. Other observations have been published from time to time, those on the horn fly have been included with muscids or dung-breeding flies in general or as incidental observations in control studies. Of these the most extensive work is that of Hammer (1941) on flies associated with pasturing cattle and that of Mohr (1943) on the ecological study of cattle droppings.



That the horn fly is an obligate blood-sucking parasite of cattle is agreed by all observers. While resting the fly chooses locations on the body of the host which cannot be reached by its head or tail. These are areas slightly behind the shoulders, at the base of the horns, middle of the belly, and low on the hind legs. In general the insects appear to seek the shaded and sheltered places on cattle (Hammer, 1941). For feeding, the back, flanks, legs and dewlap are favorite sites, or the thigh, back belly and udder when the cow is lying down. Hammer (1941) believes these are the places with thin or short hairs. Observations by McLintock (1951) in southern Alberta agree fairly closely with those of Hammer in Denmark. The feeding and resting postures of the flies are characteristically different. At rest, the wings lie nearly flat along the back and overlap at the bases, the proboscis is held nearly horizontal, and the legs are not widely separated. When feeding, the fly burrows into the hair, the wings are slightly elevated and held out from the body at an angle of  $60^{\circ}$  to the abdomen (Howard and Marlatt, 1889, 1889a; Marlatt, 1910). The legs are spread widely and the proboscis, held perpendicularly, is inserted into the hide. From 10 to 25 minutes are required for the fly to engorge itself fully (Herms, 1939, p. 339); during this time the fly withdraws and reinserts its proboscis many times with a pumping motion. Much undigested blood is discharged from the anus of the fly while in the act of feeding. The flies are intermittent



feeders (Bruce, 1942) and in the laboratory must be fed at least twice daily (Glaser, 1923). Neveau-Lemaire (1938) states that with higher temperature more blood is required by the flies.

The horn fly is about four millimeters long (Figure I), that is, about half the size of the common housefly. The mouthparts (Figure II) are essentially the same as those of the stable fly, Stomoxys calcitrans. Coloration and wing venation are much the same as that of the stable fly. Horn flies confine their attentions almost exclusively to cattle on which they remain night and day with the exception that at intervals gravid females dart down to freshly deposited cow dung for the purpose of oviposition (Glaser, 1924). The mating habits of the fly have received scant attention in the literature. Bruce (1940, 1942) states that mating has been observed as early as two days after emergence. Objects such as a lonely bush, a cart, stile, cows, or human beings were observed by Hammer (1941) to be the sites chosen for mating. The specific stimuli which induce egg deposition are unknown. The freshly passed droppings attract the flies but the attraction is not spontaneous for flies under the belly of the cow or those on the lower hind legs, when the air temperature is very high (McLintock, 1951). Mohr (1943) found that, of all the insects attracted to cow droppings, the horn fly was the only one not highly dependent upon the wind — and perhaps upon odour — for its direction of approach.



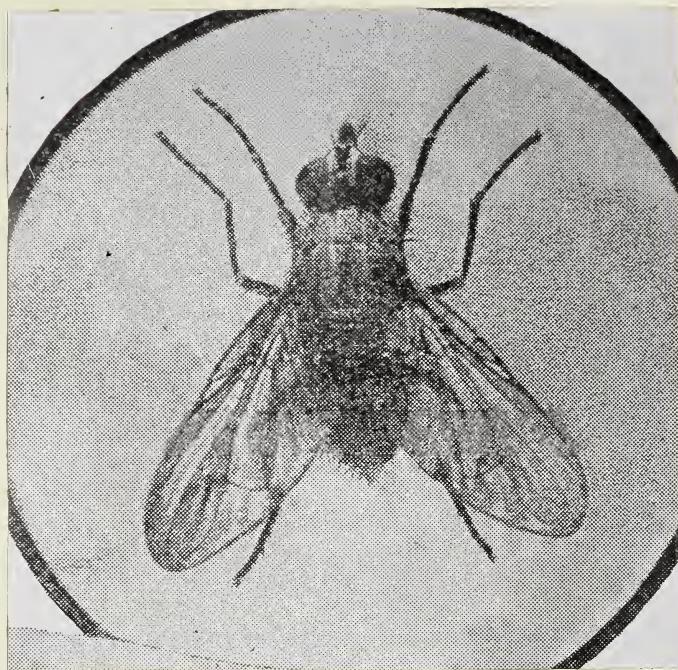


Figure I  
The Horn Fly

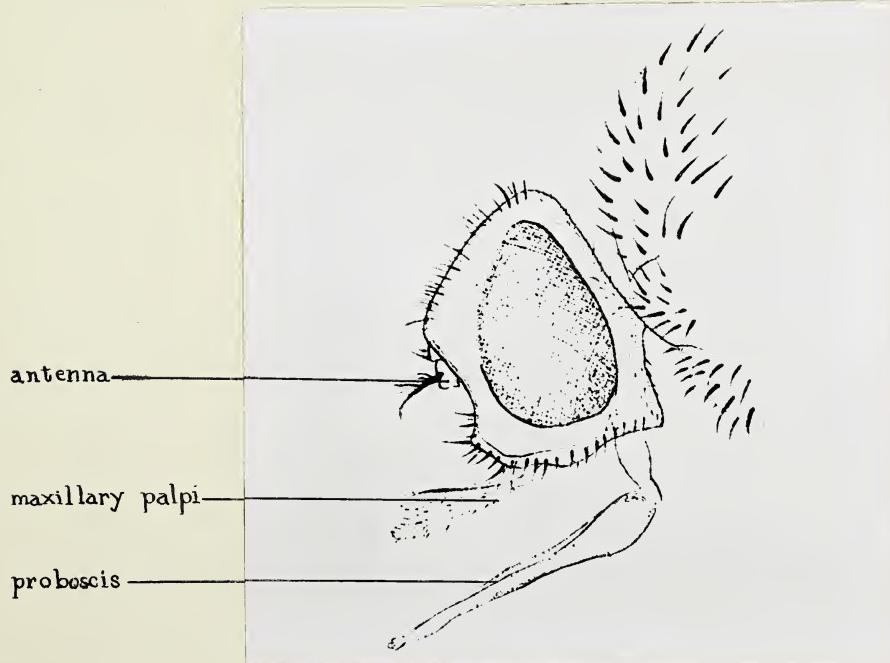


Figure II  
Mouth Parts of the Horn Fly



He believes this is due to its close presence at the time of dropping and to its conditioning to the movements of its host.

Estimates of the preoviposition time from emergence range from three days (Bruce 1940, 1942) to 17 days (Glaser, 1923). The eggs are relatively large, from 1.3 to 1.5 millimeters long, but since they are reddish brown in color they are not easily seen on the cow dung. At a temperature of 25° C the eggs hatch in 24 hours (Herms, 1939, p.338). The larvae burrow beneath the surface of the droppings reaching full growth in from three to five days. They then crawl into the drier parts and pupate. The pupal period lasts from six to eight days. The entire life cycle from egg to adult requires from 10 to 14 days at a temperature of 25° C (Figure III).

The main project arose from the desirability of controlling the horn fly population on range cattle. One of the chief problems to be considered in the general investigation was the difficulty of rearing horn flies in captivity in order to study their biological activities more adequately. Until now it has been impossible to rear these insects beyond the first generation of adults, by any known methods of artificial feeding. Observations on the habits of the fly in its natural environment suggested that the skin of cattle and/or their freshly passed droppings played some part in the fecundity of the insect: this lead to the suggestion that ingested bacteria might be essential to development



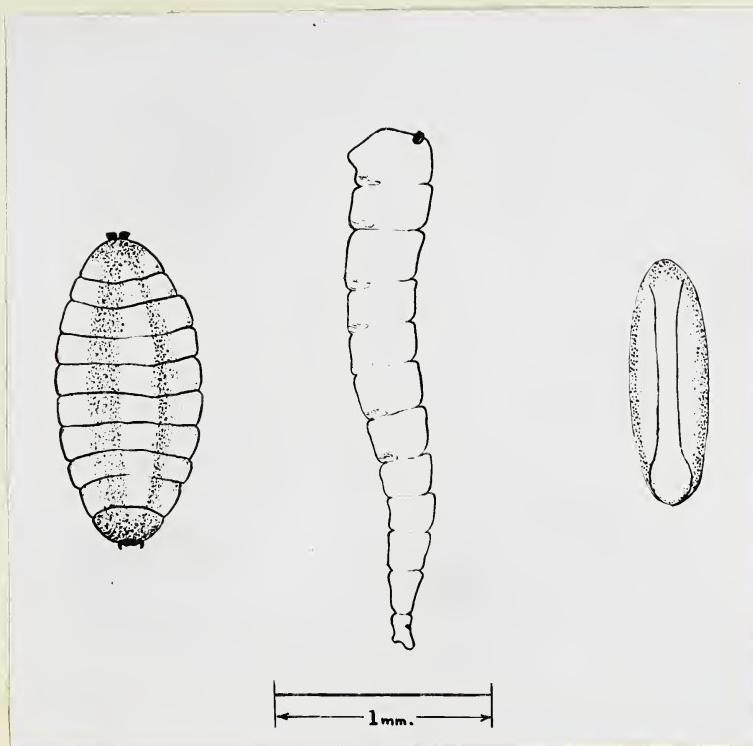


Figure III  
Ovum, Larva, and Pupa of the Horn Fly



and egg production.

The purpose of the present project is to investigate the bacteria associated with Siphona irritans in its natural environment. From this it may be possible to determine the importance of some bacteria to the life cycle of the insect. The amount of investigation which could be carried out, however, was determined largely by the supply of flies available during the limited horn fly season.



PART I. INTRODUCTION



The Importance of Microorganisms in Animal Nutrition

Just after the beginning of this century it was discovered that all kinds of microorganisms (bacteria, fungi, protozoa, spirochaetes, rickettsiae, and viruses) could be carried and transmitted by arthropods. This early work was concentrated on microbes of medical importance but during such investigations entomophilic microorganisms, unrelated to disease, were also observed. The possible importance of these organisms in the economy of insects has now become of increasing research interest.

The symbiotic relationships between mammals and microorganisms are especially important to man's economy. Symbiosis may be defined as living together and usually carries the implication that each unit concerned in the relationship derives benefit from and is necessary to the continued existence, in nature, of the associated units. No mammal is known to produce an enzyme that will change cellulose and lignin into soluble compounds, but microbes found in the rumen of cattle (Hungate, 1944, 1944a), can produce enzymes that attack these compounds and prepare them for absorption in the digestive tract. It is commonly supposed that such enzymes are formed in excess of the needs of the microorganism concerned and that the excess of the decomposition products, such as glucose from cellulose, are then available to the animal. It is possible, however, that the host has the opportunity to use these compounds coming from



the degradation of cellulose in the form in which it is built into the microbial cell. The result is the same, so far as the host is concerned, for in either case whether the enzymes are of intrinsic origin or are formed by bacteria on the material ingested the food is prepared for absorption. Lardinois et al (1944) showed that the following B vitamins were formed in the rumen of the bovine: thiamin, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, and biotin. If the animal flourishes on food not containing the full complement of vitamins then one must suppose that the deficiencies are made good through elaboration of vitamins in the digestive tract (Hastings, 1944). Products of bacterial metabolism are also important in the nutrition of other animals. Bowland (1950) showed that the addition of antibiotic supplements (aureomycin plus vitamin B<sub>12</sub>) to the diet of weakly young pigs greatly enhanced their appetite, efficiency of feed utilization and weight gain. Dam et al (1938) found that Bacillus subtilis and Escherichia coli provided vitamin K when administered to chicks fed on diets deficient in vitamin K. It has been shown by Butt and Osterberg (1938) that the vitamin K of human faeces is derived from the activity of its contained bacteria. Coates et al (1951) showed vitamin B<sub>12</sub> activity of an extract of calf faeces was approximately equal to the vitamin B<sub>12</sub> content in the Escherichia coli cells harvested from such faeces.

Microbes found in insects may be present by chance or they may be highly specialized and capable of living only



in association with the insects concerned. The microbes may cause disease or they may benefit the insect by serving as a source of food or accessory food substances. To some extent the type of association or relationship involved determines the location of the microbes with respect to the insect. According to Weber (1938) the leaf-cutting ant builds a fungus garden in which to raise its young. Usually, however, there is no such freedom of choice for the insect. Microbes may be found in the interior of the insect or on its chitinous covering. Internally the microbes are found extracellularly in the alimentary tract or in the haemocoel, or they may live intracellularly within the epithelial lining of the alimentary tract, Malpighian tubules, salivary glands, or in the cells of other tissues of the insect's body.

One of the most interesting aspects of the relationship between insects and their symbionts is the mode of transmission of these microorganisms from parent to offspring. An outstanding example of the perpetuation of extracellular bacteria through successive generations has been described by Petri (1909, 1910) in the olive fly, Dacus oleae. The bacterium Xanthomonas savastanoi, occurs in the digestive tract during all stages of the insect's development. When the olive fly deposits its egg in the fruit of the olive a quantity of bacteria from the special structures in the base of the ovipositor are also introduced and infection takes place when, upon hatching, the young larva swallows some of the surrounding bacteria. Similarly ingenious methods of



transfer occur with intracellular organisms but the modes of transmission are generally more complicated. In most cases the egg becomes internally "infected" before oviposition by way of nurse cells. For example, in the bedbug (Cimex) and in certain weevils (Apion) the symbiotes invade the nurse cells and are carried by them to the oocytes.

During the present project cultivation of intracellular microsymbiotes was not attempted. To date there are no adequate methods of culturing intracellular organisms from insects and it seems likely that if intracellular organisms were essential to the horn fly they would be inherited.

Some saprophagous insects such as the cockroach and the pomace fly depend upon a variety of fungi, yeasts, and bacteria for accessory growth factors and, in some instances at least, for their entire food supply (Brues, 1946, p.194). Within the order Diptera the fungus-gnat, Boletina, is mainly mycetophagous. Other families of Diptera (Syrphidae, Borboridae, Helomyzidae) have a similar fondness for fungi. Many such insects feed also on decaying plant and animal materials so that their diet invariably includes bacteria. The larva of the lamellicorn, Potosia cuprea, lives in the mound of the wood-ant, Formica rufra. The mound comprises pine wood and pine needles rich in cellulose which are gathered by the ants. The hind gut of Potosia contains many microorganisms, especially Bacillus cellulosam fermentans. These bacteria are thought to be of importance in the digestion of cellulose. Werner (1926) kept a lamellicorn larva alive half a year by feeding it filter paper and



expressed the belief that the cellulose must have a more important function than in building cell walls.

Roubaud (1919) asserted that the adult tsetse fly was exclusively haemophagous. The blood ingested by the fly was digested only in the middle section of the gut where the epithelial cells included symbiotic organisms. According to Roubaud, these organisms play an important part in the digestion of the blood. Wigglesworth (1929) states, however, that there is no evidence that these organisms play any part in the digestion of blood. It was pointed out independently by Wigglesworth (1929) and Aschner (1931) that symbionts occur only in those blood-sucking insects which feed solely on blood at all stages of their life cycle; these authors offered the suggestion that micro-organisms might furnish their host with some accessory food substance comparable or possibly even identical with the vitamins necessary for mammalian growth. Evidence for this hypothesis has been accumulating. It has been shown that sterile blood is an insufficient diet for mosquito larvae, but that it becomes adequate if infected with bacteria (Aschner, 1931; Rozeboom, 1935). Aschner (1932) showed that if the louse, Pediculus, is deprived of its symbionts by operative removal of the "mycetome" which contains them, its nutrition and reproductive capacity are greatly impaired, and that this impairment can be made good, to some extent at least, by the addition of yeast extract to the diet (Aschner and Ries, 1933; Ries, 1933). Hobson (1933) proved



that sterile blood is an inadequate diet for the larvae of the blow-fly, Lucilia, but that it can be made adequate by the addition of vitamin B<sub>1</sub> plus autoclaved extract of yeast.

It has been demonstrated that bacteria or bacterial products may be important for the continued existence of many insects throughout their entire life cycle. Bacteria can produce proteolytic, lipolytic, saccharolytic and other enzymes which have considerable influence on the digestion of the insect harboring them. Petri (1905) was the earliest to assign bacteria a definite role in the digestion of an insect. He found that the bacteria, constantly in the caeca of the olive fly, produce lipase. Brown (1928) found that most of the digestive enzymes of the honey bee, Apis mellifera, were also produced by the microorganisms present in its digestive tract. These enzymes included invertase, both peptic and tryptic proteolytic enzymes, and a lipase. In addition to this effect on the nutrition of adult insects bacteria and their products may play an important part in the continuation of an insect's life cycle. Hinman (1930) found that the larvae of the mosquito, Aedes aegypti, failed to develop in sterile synthetic media or in autoclaved water taken from the normal breeding places. Certain bacteria added to the water made it suitable for larval development. Rozeboom (1935) found that some bacteria used to feed the larvae of the mosquito were more suitable than others. Organisms found in the normal breeding places were the most suitable: Bacillus subtilis, Bacillus mycoides, Escherichia



coli, Aerobacter aerogenes, and Pseudomonas fluorescens were of equal value but Sarcina lutea was negligible in its effect. On the other hand organisms such as Pseudomonas aeruginosa rapidly killed the larvae. Glaser (1924) suggested that the growing larvae of the housefly, Musca domestica, was dependent upon accessory growth factors from bacteria and yeasts, but later demonstrated that this dependence was not absolute. In 1938 he succeeded in raising houseflies on nutrient medium free from microorganisms. *Drosophila* and other fruit flies, living in an environment heavily populated with yeasts, bear these minute plants abundantly on their bodies. There is a constant relationship between the presence of yeasts and the development of the flies eggs. Apparently the yeast fermentation is of importance in providing a suitable food supply for the larvae.

In this portion of the thesis, an attempt has been made to review selectively some of the more important reports indicating the undeniable significance of intestinal micro-organisms in the nutrition of the host.

This recorded data favors the hypothesis that bacteria, and particularly enterophilic bacteria, may have some influence on the fertility of Siphona irritans. The difficulties of rearing horn flies in artificial conditions may be related to the absence of some factor, possibly bacterial in origin, supplied under natural conditions from the flies environment.



PART II. MATERIALS AND METHODS



Collection, Handling, and Rearing of Siphona irritans

Wild horn flies were collected from cattle at the Lethbridge Stockyards, from cattle at the Lethbridge Experimental Station, and occasionally from herds at different localities in southern Alberta. The flies were caught by sweeping the backs or bellies of the cattle with a net and transferring them to cotton plugged Erlenmeyer flasks. Early in the investigation the flies were caged and shipped alive to Edmonton on the same day that they were captured. Later, when laboratory reared flies were required at Lethbridge, the wild flies were placed over fresh cow manure in battery jars for 24 hours. No food was supplied to the flies and during the 24 hours their eggs were deposited on the manure and around the walls of the jars. At the end of the 24 hours those flies which had survived were shipped to the Department of Bacteriology of the University of Alberta in Edmonton. For shipment to Edmonton the flies were transferred by means of an aspirator to cylindrical wire cages. These cages were 5 1/2 inches high and 4 1/2 inches in diameter, with the wall made of 20-mesh galvanized iron screen. One end of the cylinder was covered with light cotton and the other end was closed with a light cotton sleeve. In transit or storage the sleeve was tied into a simple knot to prevent the flies from escaping. Before each shipment of flies the cage was autoclaved to prevent contamination from earlier batches. Each cage received up to 675 flies. For shipment a card-



board carton five inches square and six and a half inches high lined with heavy waxed paper was used. A pad of moist cellucotton was next placed on the bottom of the carton and the cage placed on top of the pad. The carton was then closed and sealed with kraft tape. The cartons were shipped the 340 miles to Edmonton by railway or air express. Only an occasional fly was dead on arrival at Edmonton.

In the Livestock Insect Laboratory at Lethbridge flies were reared from eggs obtained from the wild females. If the manure used appeared to be very wet, a two-inch layer of sand was first placed in the bottom of the battery jar before the manure was added. When the manure was comparatively dry no sand was used. Eggs which were laid on the manure by the wild females were left undisturbed to hatch and the larvae allowed to complete their larval development in the jars. Eggs which were laid in the Erlenmeyer flasks were washed out with water and transferred to the filter paper in a Buechner funnel. After the excess water had been drawn off by suction the eggs were brushed onto the surface of fresh manure in battery jars. These battery jars with their contents were kept in a cabinet at about 25° C. At this temperature the eggs hatched in less than 24 hours and the larvae completed their development in about six days. When sand was used in the battery jars the puparia were found in the sand and could be removed by adding the sand to water and stirring. The puparia floated to the surface of the water and could be removed with a small strainer. When no



sand was used the puparia were found in the drier parts of the manure just under the crust. The crust was removed and the manure underneath, slightly moist and caked, was emptied from the battery jar and broken into a Waring blender. Water was then added and the whole agitated slowly. After the manure settled the puparia were removed with the strainer. Washing the puparia in water was also necessary in order to remove parasitic mites. The puparia were distributed in Petri plate covers which were placed in the bottom of a cage described by McLintock (1952) where the flies were allowed to emerge. At 25° C emergence began in about five days after formation of the puparia. Since the females began to emerge first, the flies were not removed from the emergence cage until a day or two had elapsed since the beginning of the emergence.

The flies, in groups of 50, were then placed in sterile lantern chimneys. The top of each chimney was closed with a single layer of surgical gauze and each chimney set on an inverted Petri plate cover. Daily the flies were given a single meal of defibrinated beef blood containing approximately 2.5% sucrose added as a 10% solution. The blood and sugar mixture was offered to the flies soaked up in thin, sterile, gauze pledges about one inch square. These pledges were placed on top of the gauze covering the chimneys and each covered with a sterile watch glass. It was impossible to follow a strict sterile technique in administering the diets but a close watch was kept to insure that contamination was at a minimum. Two experiments, employing the above described



feeding method plus specific bacterial organisms added to the diet were carried out; these experiments will be described in a later section.

On their arrival in Edmonton wild and laboratory reared flies were counted and divided into lots for experimentation. Bacteriological examination began on the same day the specimens arrived at their destination. The flies were transferred to a large sterile test tube by placing the open end of the tube into the cage through the untied cotton sleeve. Horn flies are attracted to light and this phototropic response was used in removing the flies from the cage. The cage was covered so as to darken the interior and a bright light placed against the closed end of the test tube protruding from the sleeve. When the desired number of horn flies had been attracted into the tube it was withdrawn and plugged immediately with sterile cotton. The cotton sleeve was then retied. Living flies were removed from the test tube one at a time with the aid of sterile forceps. A red hot needle was placed against the head of a fly killing it almost instantaneously. By this method wild and laboratory reared horn flies were prepared for the examinations which will be described subsequently.



Isolation of Microorganisms  
from the External Surfaces of Siphona irritans

Seven lots comprising a total of 72 wild horn flies were examined for external microorganisms. The number in each lot varied from 5 to 27 flies with four lots containing five flies each. Each lot of flies was placed in a test tube containing about five milliliters of nutrient broth (see Appendix) and incubated at room temperature or at 37° C for 24 hours. The resulting bacterial growth, as evidenced by clouding of the broth, was subcultured onto nutrient, enriched, and selective media. The inoculum was distributed evenly over the surface of each medium, contained in a Petri plate, with a sterile glass spreader. The media used for isolating microorganisms from each broth tube were: one nutrient agar plate incubated at room temperature: two blood agar plates incubated at 37° C for 24 hours, one plate aerobically and the other anaerobically in a McIntosh and Fildes' jar: one MacConkey's agar plate incubated at 37° C for 24 hours: and one Littman's agar plate incubated at room temperature for one month (this medium is for the isolation of yeasts and fungi). Each colony of microorganisms, on each of these plates, having a morphology distinct from the others present was picked to a nutrient agar slant. Each colony that produced haemolysis on blood agar was picked to a Loeffler's serum slant. The composition of all the media mentioned above is described in the Appendix.



A smear of each colony of organisms was made immediately after the organisms were picked to a slant. The material was suspended in a drop of sterile water and spread uniformly on a clean glass slide. The smear was then fixed in a Bunsen gas flame and stained by the Hucker modification of Gram's stain (Manual of Methods for Pure Culture Study, p. IV<sub>51-8</sub>). Storage of the pure cultures was in a refrigerator at 6° C. Identification of these organisms and tests on them were carried out at a later date. The above described procedure for the isolation of microorganisms was employed throughout the entire project.



Selection of a Suitable Disinfectant  
for the External Surfaces of Siphona irritans

In order to study the microorganisms solely within the fly it was necessary to eliminate microorganisms on the outside of the fly. Two chemicals, merthiolate and bichloride of mercury, were tested. Each was prepared in a 1:500 solution — the former in 50% ethanol and the latter in 70% ethanol. A 1:1000 solution of each disinfectant was obtained by making appropriate dilutions. The fly was left in the disinfectant for 15 to 30 minutes and then washed in sterile physiological saline (see Appendix) for two ten minute periods. Washing the fly in saline served to remove the disinfectant. The treated flies were placed in test tubes containing nutrient broth in lots of five to ten flies and incubated at 37° C for 96 hours. At the end of this time bacterial growth was evidenced in clouding of the broth. From Table I it will be noted that the disinfecting action of bichloride of mercury was variable. This may be explained, in part, in that the disinfectant was restricted from penetrating into the tracheae, due to increased surface tension, thus allowing some of the microorganisms present there to escape the action of bichloride of mercury. Merthiolate gave variable results with the number of flies tested. It is probable that the tracheae also harboured a number of organisms untouched by the merthiolate. Since one disinfectant seemed as effective as the other merthiolate was employed during the preliminary



work because of the short period during which wild flies were available and the urgency of other experimental work. During the first experiments it was observed that Orvus, a detergent, when added to the merthiolate solution seemed to be of value in decreasing surface tension on the fly. The merthiolate solution was saturated with the detergent. Flies treated with the detergent were observed under a binocular microscope to have a decreased number of air bubbles on their surfaces. The merthiolate could then act on a greater surface area of the fly.



Table I

Testing Bichloride of Mercury

and Merthiolate as Disinfectants on Horn Flies

Disinfectant	No. of flies treated	Minutes in Disinfectant	Minutes in phys. saline	No. of colonies
Bichloride of mercury	10	20	1-2	+++
	5	30	30	+
	5	15	1-2	o
Merthiolate	8	20	1-2	+++
	8	30	1-2	++
	8	30	1-2	+
	10	25	25	++

Symbols:

"+++"represents a large number of colonies and

"o" represents the absence of colonies on a streak

plate subcultured from the broth.



The use of Thioglycollate Medium  
to detect possible flaws in the Disinfectant procedure

The purpose of this portion of the investigation was to determine whether or not the technique of rinsing the mercurial solution from the fly with physiological saline was adequate. If the disinfectant was not removed from the fly during the rinse it could act upon organisms liberated from the interior of the fly when it was crushed for culture. The disinfectant might exercise a bactericidal or a bacteriostatic effect on the internal organisms but in neither case would they grow in culture. Brewer (1939) suggested that the addition of sodium thioglycollate to media interfered with the bacteriostatic action of mercurial disinfectants and allowed those organisms which were not killed to resume growth. Nungester, Hood, and Warren (1943) demonstrated that the optimum concentration of sodium thioglycollate for inhibiting the bacteriostatic effect of mercurial compounds was between 0.10% and 0.05%. In this investigation 0.085% sodium thioglycollate was added to nutrient broth. The mercurial compounds tested were merthiolate and bichloride of mercury, both in concentrations of 1:500 and 1:1000. An intact fly was placed in 20 milliliters of disinfectant contained in a sterile Petri plate. After 15 minutes the fly was removed from the disinfectant with a pair of sterile forceps and placed in a physiological saline rinse for two ten minute periods. The fly was then transferred to a test



tube of nutrient broth containing 0.085% sodium thioglycollate. Another fly, which had been exposed to disinfectant and rinsed in the same manner, was placed in a test tube of nutrient broth as a control. Both tubes were incubated at 37° C for 96 hours. The results summarized in Table II indicate that the percentage of positive growths was essentially the same whether or not sodium thioglycollate was used. From this it may be assumed that the physiological saline rinse was removing most of the mercurial compound or that it was at least reducing it to a non-inhibitory concentration.



Table II

Tests to Determine the adequacy  
of the Physiological saline rinse

Disinfectant	Concen- tration	No. tests	No. with positive growths	
			Sodium thio- glycollate broth	Nutrient broth control
Merthiolate	1:500	31	28	30
	1:1000	31	29	31
Bichloride of Mercury	1:500	31	16	21
	1:1000	31	19	19



Further Problems in Disinfection  
of the External Surfaces of Siphona irritans

When sterilizing the external surfaces of the fly it was necessary to prevent the disinfectant from coming in contact with the inner organs of the insect. For this reason it was necessary to kill the fly and yet keep its body intact. At the beginning of this investigation a living fly was placed directly into the disinfectant. By this method the fly was killed either by the toxicity of the disinfectant, or by the inability of the fly to obtain oxygen through the tracheae because of blocking by the fluid, or a combination of toxicity and smothering. The fly often struggled for a long period of time when placed in the disinfectant and it was apparent that portions of the fly, especially the dorsal surface, were not receiving disinfectant adequately. The activity of the fly was reduced considerably when the wings were clipped off at their bases with a pair of scissors but this method was time consuming and therefore was not used extensively. An alternative method of killing the fly with a red hot needle was found to be the most suitable and it was used the most often. After the fly was killed it was transferred immediately to the disinfectant and left there for 15 minutes. It was unlikely that the disinfectant penetrated into the inner organs through the small rupture in the cuticle of the head. If any disinfectant did pass through the rupture it seemed unlikely that after death any further adsorption would take place. In the experiments



described subsequently no evidence was obtained of any interference with the viability of internal microorganisms. After treatment with the disinfectant the fly was rinsed in physiological saline for two ten minute periods. With this technique there was no evidence that the disinfectant penetrated into the deep organs or affected microorganisms which might be present there.



Isolation of Microorganisms  
from the Interior of Siphona irritans

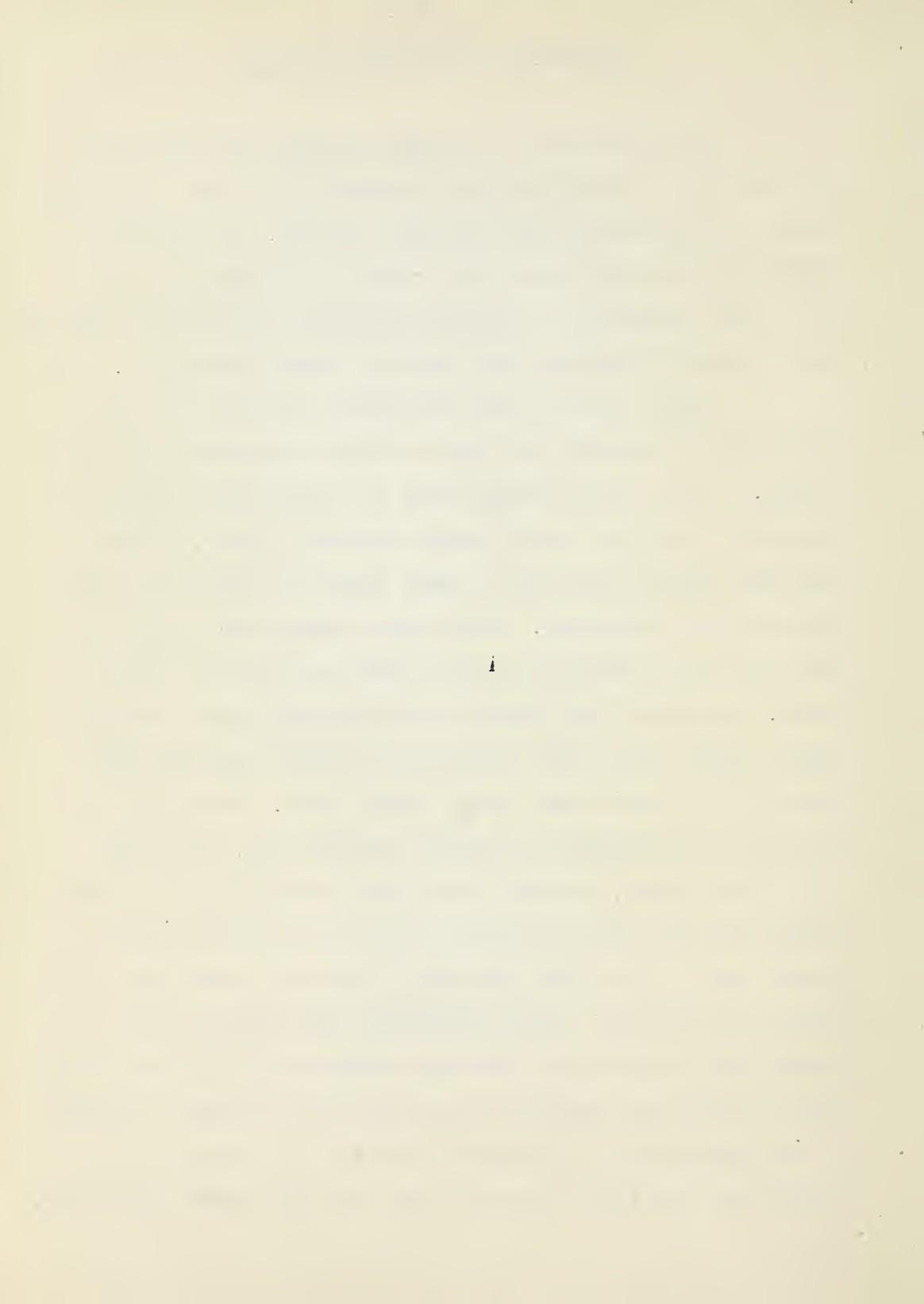
The external surfaces of the fly were sterilized by the method described (page 31). The insect was then transferred aseptically to a test tube containing nutrient broth and the whole incubated at 37° C for 96 hours. If there was no bacterial growth, as evidenced by the absence of cloudiness in the broth after incubation, the fly was crushed against the side of the tube with a sterile glass rod. The broth was incubated at 37° C for 24 hours and the bacterial growth subcultured on solid media (page 23) in order to obtain individual strains of bacteria. Bacteria isolated by this method were assumed to be largely from the digestive tract but one cannot overlook the fact that a number of organisms in the tracheae undoubtedly escaped disinfection. A more detailed study was made on bacteria obtained directly from the dissected out lower digestive tract as shown in the next section. Organisms were also isolated from the interior of ova, larvae and pupae by the method described above.



Dissection of Siphona irritans

The dissection of Siphona irritans was necessary in order that a microbiological examination of the intestinal tract and interior of the fly might be made. The exterior of the fly was sterilized with 1:1000 merthiolate solution or a 1:500 bichloride of mercury solution as described (page 25) and an aseptic technique was employed during dissection.

After removal from the saline rinse the fly had to be dried externally so that it could be properly embedded in wax. No one method seemed best but immersion in ether or alcohol followed by rapid passage through a flame, offered the most rapid method when a small number of flies was being prepared for dissection. When large numbers were dissected the flies were placed on sterile blotting paper in a Petri plate. Drying by this method took approximately 45 minutes during which time it was necessary to replace the blotting paper two or three times as it became moist. When each fly was dry it was embedded in melted paraffin wax, contained in a Petri plate, in such a way that the fly could be dissected from the ventral surface; that is, it was embedded dorsal surface down with one half of the body above the surface of the wax. Before embedding, the paraffin was heated for 15 minutes to destroy contaminating microorganisms. After the fly was embedded and the paraffin allowed to harden, a sufficient amount of Ringer's solution (see Appendix) was poured into the Petri plate to cover the fly during dissection.



This solution was used to help maintain the osmotic pressure of the cells and to prevent the tissues from drying out during dissection. For actual dissection a Bosch and Lomb low power binocular microscope, a good source of light (100 watt white light lamp), and dissecting instruments were necessary. The latter consisted of sharply pointed forceps, a pair of fine scissors, scalpels, and needles. A dissecting needle consisted of a fine needle bent in the middle at a 90° angle and fixed into a glass rod handle. This needle was found most useful because of its manoeuverability and because the operator could steady his arms on the bench during dissection. The abdomen of the fly was opened on the ventral surface by making an incision from the constriction of the thorax to the anus with a scalpel and a pair of fine scissors. The abdominal viscera were extracted through the cuticle with the forceps and transferred to nutrient broth for culture. When the digestive tract alone was to be removed the abdomen was opened as described above and two dissecting needles used to tease away the viscera so as to lay the gut bare. The upper intestinal tract was removed from the thorax by holding the ileum with a pair of forceps and pulling the gut down. An incision through the constricted portion of the rectum was made to detach the gut from the fly. The gut was then transferred with forceps to a test tube of nutrient broth and the gut crushed against the side of the tube with a glass rod. The broth was then incubated at 37° C for 24 hours and the resulting growth subcultured on the solid media described (page 23).



A Method for the rapid removal of Intestinal Tract

The large number of flies to be examined in the course of this work necessitated the search for a technique both simple and quick. After considerable trial the following technique was adopted. A living fly was killed with ether and placed dorsal surface downward in melted paraffin contained in a Petri plate. The paraffin was about one-sixteenth of an inch in depth which left the ventral surface of the fly exposed above the surface of the wax. When the paraffin hardened 1:500 bichloride of mercury solution was poured into the plate and allowed to act from one to five minutes. The disinfectant was poured off and the flies rinsed for two ten minute periods with physiological saline. During dissection under a binocular microscope the flies were covered with Ringer's solution. The microscope was swabbed with 1:500 bichloride of mercury solution just prior to use and the dissection was carried out in a room where air currents as a source of contamination were negligible. Dissecting instruments, scalpel and sharply pointed forceps, were sterilized in a boiling water bath for twenty minutes just before use and were returned to the bath during dissection when not in use. To remove the viscera an incision was made with a scalpel from the constriction of the thorax and abdomen to the anus. The scalpel was then slipped through the anterior portion of the incision, under the exoskeleton, along the left side of the abdomen and down under the viscera. The



scalpel was then raised so as to remove the viscera from the abdomen. An incision through the rectum and oviducts or vas deferens freed the viscera from the fly. Fat bodies, ova, and other internal structures were teased away from the intestinal tract with two sterile dissecting needles. The tract was then transferred with sterile forceps into a tube of nutrient broth and crushed against the side of the test tube with a sterile glass rod. The broth was used to wash the material from the side of the tube.



Preparation of Faecal smears

Faecal smears were made in order to determine whether or not all organisms present in the gut of the fly were growing on subcultures and also to increase the certainty that the organisms appearing on subculture were derived from the gut. Living flies captured in their natural habitat were transferred from the wire shipping cage to a large clean test tube. The test tube was plugged with cotton and five milliliters of ether ~~poured~~ into this plug in order to kill the flies. After five minutes the plug was taken out of the tube. Flies were removed singly from the tube with a pair of sterile sharply pointed forceps and placed on a clean glass slide with the ventral surface of the fly downward. The slide and fly were placed under a low power binocular microscope. The forceps were then placed along the abdomen of the fly with each arm of the forceps lateral to the abdomen. The abdomen was squeezed gently with the forceps until the watery faecal material passed from the anus. A sterile platinum wire was then used to spread this material uniformly over the slide in an area of from five to ten millimeters in diameter. The smear was then fixed in a Bunsen flame and stained by Gram's method.



Preparation of Histological Sections

Histological sections of wild horn flies were made in order that a microscopic examination could be carried out for microorganisms which might be present within the fly. Of 45 flies selected at random during the fly season and intended for sectioning eight were finally suitable for microscopic examination. The reason for the small number of suitable flies obtained will be described subsequently. Multiple pricks, employing a fine needle, were made in each fly before it was placed in Carnoy's fixative (see Appendix) and before introduction into each of the solutions thereafter, in order to allow the penetration of fluid through the chitinous exoskeleton. Only those flies which settled to the bottom of the fixative solution and the following solutions were sectioned; flies which remained at the surface of the fixative or any one of the following solutions were discarded. This explains the small number of suitable flies finally obtained. For fixation the fly was placed in Carnoy's fluid at approximately 50 to 55° C overnight followed by two changes of absolute ethanol in five hours and two changes of chloroform in three hours. The flies were then left overnight in chloroform and paraffin (1:1) at 50 to 55° C and then changed in paraffin three times in six hours and finally embedded in paraffin blocks. Serial sections cut with a microtome at six microns and stained by the Glynn modification of Gram's method (Glynn, 1935) were found to show up internal structures satisfactorily. Microscopic examination of the



serial sections revealed that bacteria were present only within the lumen of the lower digestive tract. No special cells resembling mycetomes were seen. The numbers of organisms present varied but the guts of all of the flies examined contained either small Gram negative rods or Gram positive cocci in clusters, or both together.



Testing for Antagonistic action  
among organisms isolated from Siphona irritans

As the bacteria present in the intestine of wild horn flies were isolated and identified it was apparent that certain genera, namely Achromobacter, Alcaligenes, and Micrococcus were the organisms appearing most consistently. Out of this arose the suggestion that bacteria comprising the normal flora of the intestine (page 83) might exert an antagonistic action on other organisms occurring there fortuitously. To test this hypothesis representative organisms isolated from the gut of the fly were selected. Organisms representative of the normal flora of the gut included: genus Achromobacter (four strains were employed), genus Alcaligenes (two strains), Micrococcus candidus, Micrococcus epidermidis, and Micrococcus flavus. Adventitious organisms (page 83) occurring in the gut were: genus Bacillus (two strains), Bacillus brevis, and four unidentified organisms. In testing for antagonistic action the following technique was employed. An organism of the normal flora was inoculated on a nutrient agar slant and incubated at 37° C for 24 hours so that actively growing young organisms could be obtained. The organism was then inoculated diametrically across a blood agar plate in a single streak. The plate was then incubated at 37° C for 24 hours. Individual streaks of adventitious organisms, also young and actively growing, were then made perpendicular to the first streak and the plate incubated at 37° C for 24 hours (Figure IV).



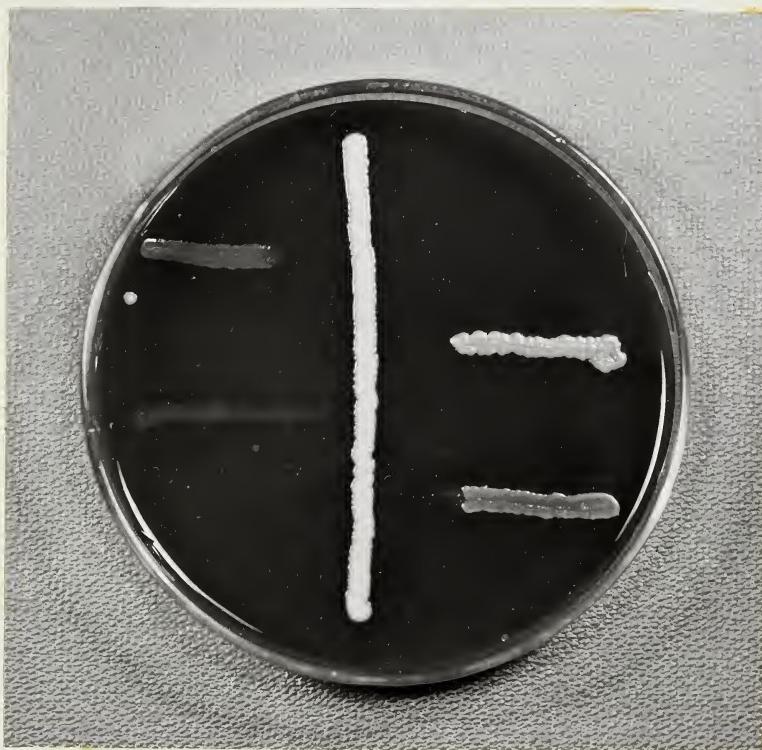


Figure IV

Antagonism of Micrococcus epidermidis against adventitious organisms isolated from the digestive tract of the horn fly.



Any evidence of growth inhibition was checked by a repeat test before being accepted as authentic. From Table III it can be concluded that M. epidermidis and M. flavus exert a pronounced inhibitory effect on the growth of most adventitious organisms occurring in the intestinal tract of the fly. One bacterial strain in each of the genera Achromobacter and Alcaligenes exerted variable inhibitory action<sup>on most</sup> of the adventitious organisms.

Tests were made to determine whether organisms comprising the normal intestinal flora of Siphona irritans have an antagonistic action on each other. The technique employed was the same as that described (page 41). The results of these tests are shown in Table IV. Culture B<sub>125</sub><sup>b</sup> of the genus Achromobacter had an inhibitory action on the growth of culture B<sub>127c</sub> of the same genus, and a less inhibitory action on M. epidermidis and M. flavus. Culture B<sub>163</sub><sup>a</sup> of the genus Alcaligenes had a slight inhibitory action on culture B<sub>127c</sub> and B<sub>135</sub><sup>b</sup> of the genus Achromobacter. M. epidermidis had a pronounced inhibitory action on culture B<sub>125</sub><sup>b</sup> of the genus Achromobacter. All results were confirmed by repeated tests. From Table IV it can be concluded that there is weak antagonism among some organisms comprising the normal intestinal flora. This suggests that the intestinal organisms of the horn fly tend to keep their own numbers in check by a weak antagonistic action.

Toward the end of the 1950 fly season it was observed that an increasing number of Gram negative rods



appeared in cultures made from wild horn flies. This alteration in intestinal flora coincided with the decreasing activity of flies toward the end of the season and might even have been contributory. No evidence of this nature can be produced, but it seemed interesting to find out if the change was due to any endogenous bacterial activity. A tentative experiment along the preceding lines showed (Table V) that Escherichia freundii exerted an inhibitory action on all the cocci except M. epidermidis. This antagonistic action was very weak. Among the other organisms tested there was no evidence of antagonism. The Gram negative organisms tested did not significantly inhibit the cocci and the alteration in bowel flora was not caused by any new and potent bacterial antagonism.



Table III

Antagonism of organisms comprising the normal intestinal flora of *S. irritans* against adventitious organisms.

Normal flora organism	Adventitious organism						
	Bacillus B <sub>14</sub> <sup>a</sup>	Bacillus B <sub>196</sub> <sup>b</sup>	Bacillus brevis B <sub>33</sub> <sup>a</sup>	Unidentified organism B <sub>47</sub> <sup>a</sup>	Unidentified organism B <sub>48b</sub>	Unidentified organism B <sub>48a2</sub>	Unidentified organism B <sub>136c</sub>
Achromobacter B <sub>125</sub> <sup>b</sup>	o	+	++	+++	++	o	++
Achromobacter B <sub>127</sub> <sup>c</sup>	o	o	o	o	o	o	o
Achromobacter B <sub>132</sub> <sup>a</sup>	o	o	o	o	o	o	o
Achromobacter B <sub>135</sub> <sup>b</sup>	o	o	o	o	o	o	o
Alcaligenes B <sub>27</sub> <sup>b</sup>	o	o	o	+	o	+	+
Alcaligenes B <sub>163</sub> <sup>a</sup>	+	+	+	+	+	o	o
M. candidus	o	o	o	o	o	o	o
M. epidermidis	++++	+++	+++	++++	++++	+	+++
M. flavus	++++	++	+++	++++	++++	++	++++

Symbols:

"o" indicates the absence of antagonistic action against an adventitious organism

"+++" indicates a high degree of antagonism against an adventitious organism and "-" a low degree of antagonism.

\*Unidentified organism No. B<sub>47a</sub> is a large Gram positive anaerobic rod; B<sub>48b</sub> and B<sub>48a2</sub> are large Gram positive aerobic rods; and B<sub>136c</sub> is a large Gram negative aerobic rod. A further description of each of these unidentified organisms is given (page 72).



Table IV

Antagonism of organisms comprising the normal intestinal flora of *S. irritans* on each other.\*

	Organism Tested	Achromobacter B125b	Achromobacter B127c	Achromobacter B132a	Achromobacter B135b	Alcaligenes B27c	Alcaligenes B163a	Micrococcus candidus	Micrococcus epidermidis	Micrococcus flavus
Antagonist										
Achromobacter B125b		++	o	o	o	o	o	o	+	+
Achromobacter B127c	o	o	o	o	o	o	o	o	o	o
Achromobacter B132a	o	o	o	o	o	o	o	o	o	o
Achromobacter B135b	o	+	o	o	o	o	o	o	o	o
Alcaligenes B27b	o	+	o	o	o	o	o	o	o	o
Alcaligenes B163a	o	+	o	+	o	o	o	o	o	o
M. candidus	o	o	o	o	o	o	o	o	o	o
M. epidermidis	++++	o	o	o	o	o	o	o	o	o
M. flavus	o	o	o	o	o	o	o	o	o	

\*The symbols used in Table IV have a similar connotation to those used in Table III. In each case they indicate the effect of the "antagonist" on the test organism.



Table V

Antagonistic action between Gram negative rods  
and Gram positive cocci isolated from S. irritans.\*

Organism Tested	<i>M. caseolyticus</i>	<i>M. epidermidis</i>	<i>M. flavus</i>	<i>M. luteus</i>	<i>E. coli</i>	<i>E. intermedium</i>	<i>E. freundii</i>	<i>A. aerogenes</i>	<i>P. rettgeri</i>
<b>Antagonist</b>									
<i>E. coli</i>	o	o	o	o					
<i>E. intermedium</i>	o	o	o	o					
<i>E. freundii</i>	+	o	+	+					
<i>A. aerogenes</i>	o	o	o	o					
<i>P. rettgeri</i>	o	o	o	o					
<i>M. caseolyticus</i>					o	o	o	o	o
<i>M. epidermidis</i>					o	o	o	o	o
<i>M. flavus</i>					o	o	o	o	o
<i>M. luteus</i>					o	o	o	o	o

\*The symbols used in Table V have a similar connotation to those used in Table III. In each case they indicate the effect of the "antagonist" on the test organism.



Isolation of Microorganisms from  
the Natural Environment of S. irritans

In its habits the horn fly, Siphona irritans, is distinct from all other blood-sucking diptera which attack cattle in that it remains almost constantly on its host. Because of this habit it was suggested that the skin of cattle played some part in establishing the insect's fecundity and it was considered necessary to make a study of micro-organisms on the skin of cattle. Accordingly swabs were taken at the Lethbridge Experimental Station Dairy Barn and submitted to Edmonton by rail and air express. The hide surfaces swabbed were those covering the pastern, hock, and pin bones (Sisson, 1914, p.898). Horn flies are often seen to rest on these areas, particularly just before they move to droppings to deposit their eggs. The pastern is defined as that part of the foot of the horse and allied animals between the coffin joint and fetlock. Swabs were taken from the flexor surface of the pastern. The hock is defined as the tarsal joint in the hind limb of cattle corresponding to the ankle of man, but elevated and bending backwards. The swabs were taken at the point of the hock. Pin bones are defined as the hip bone or innominate bone of animals such as cattle. Swabs were taken from the area between the pin bones. Swabs were taken from six cattle in September, 1951, and shipped to Edmonton on nutrient agar slants and in special anaerobic transport medium (see Appendix). Two swabs were



also taken from the hide on the back of a calf on which laboratory reared flies had been fed. These flies had shown considerable development of ovaries. Each swab was used to inoculate solid media and organisms were isolated as described (page 23).

The fly leaves its host only for a matter of minutes to deposit its eggs on freshly passed cow dung. In doing so it moves over the surface of the fresh cake. This suggested that fresh cow droppings played some part in establishing the insect's fecundity. Faecal samples and perianal swabs were collected in June, 1950, at Lethbridge and submitted to Edmonton by railway and air express. Twelve stool specimens and four perianal swabs were examined bacteriologically. Each of the specimens was plated and organisms isolated by the method described (page 23).



Classification of Microorganisms

The criteria for fungi, yeast, and mold identification are based on the recommendations of Engler and Prantl (1900), Thom (1930), Gilman (1945), and Thom and Raper (1945). Identification was restricted to morphological characteristics on nutrient agar and is therefore only tentative.

The criteria for bacterial identification are based on the recommendations of Bergey's Manual of Determinative Bacteriology, 1948, Sixth Edition. The recommendations include a detailed study of an organism in regard to its morphological, cultural, physiological, and pathogenic characters; but such completely detailed studies were not considered necessary to identify organisms isolated during the present project. Only significant characters which could be used to identify with accuracy the organism as far as genus, or genus and species, were studied. The composition of each of the media employed is given in the Appendix. Observations on inoculated media were made after 24, 48 hours, 1, 2, and 3 weeks incubation. Examples of the method of classification are given below:

An organism which is a Gram positive coccus liquefies gelatin slowly, acidifies litmus milk, produces nitrites from nitrates, usually does not haemolyze blood, and grows well aerobically at 37° C is identified as Micrococcus epidermidis according to the classification on page 236 of Bergey's Manual.



An organism with the dry and wrinkled colonial morphology of a spore bearer but showing no spores in the original smear was inoculated into nutrient broth and incubated at room temperature, 25° C, from five to seven days. A smear was made, stained with methylene blue, and examined for spores. All the spore bearers were found to be catalase positive and were classified into the genus Bacillus. Some of these organisms were classified into species with the aid of certain biochemical tests listed on pages 706 and 707 of Bergey's Manual.

A Gram negative organism was inoculated into peptone water, and sugar broths (lactose, dextrose, sucrose, and mannite). If the organism produced acid and gas from lactose and dextrose within 24 hours at 37° C it was classified into the Tribe Eschericheae. It was then classified into genus and species according to the cultural characteristics listed on pages 444 and 445 of Bergey's Manual. An organism which did not ferment lactose and dextrose within 30 days at 37° C was inoculated into urea medium. The organism was classified into genus or genus and species according to the cultural characteristics listed on page 486 of Bergey's Manual.

A Gram negative organism which did not decompose urea within 48 hours was inoculated into nutrient broth and incubated at 30° C for 24 hours. A test for motility was made either by the hanging drop method or by placing a drop of the 24 hour broth culture on a clean glass slide and placing a cover slip over it. Motility was observed with the



high power dry objective of the microscope. If the organism was motile it was inoculated into a test tube containing soft agar (see Appendix) to obtain organisms with good flagellar structure. The test tube contained a glass cylinder five centimeters long with a four millimeter bore. The soft agar had a depth of about 25 millimeters and the glass cylinder stood perpendicularly in the test tube with about 25 millimeters of it above the surface of the soft agar. The motile organism was inoculated into the medium within the cylinder and incubated at 30° C for 7 to 48 hours. The most actively motile organisms moved down the cylinder, through the opening at the bottom of it, and up to the surface of the surrounding medium. When the organism arrived at the surface of the medium it was "picked" and then inoculated onto a nutrient agar slant and incubated at 30° C for 18 hours. Kirkpatrick's method (Browning and Mackie, 1949, p.786) was used in preparing and staining a smear of the organism. Microscopic examination revealed that all the organisms had four or more peritrichous flagella. These motile organisms and other non-motile organisms, not belonging to the groups previously described, grew on MacConkey's medium. They produced little or no acid in litmus milk medium. Some organisms exhibited yellow chromogenesis. The organisms were classified into the Family Achromobacteriaceae. Their cultural characteristics on agar, litmus milk, and sugar broths were used to classify them into the genera listed on page 412 of Bergey's Manual.

A number of small, moderate, and large size Gram



positive and negative rods which were not found to belong to any of the above described genera were isolated. Their cultural, physiological, and morphological characteristics are given (Part III).



Some tests used in the identification of Bacteria

For the composition of media and reagents mentioned below consult the Appendix.

An organism, being tested for its ability to produce acetyl methyl carbinol, was inoculated into buffered dextrose broth and incubated at 37° C for 24 hours. One milliliter of this was pipetted into another test tube. Four drops of 40% NaOH and 12 drops of alpha-naphthol solution were added. The whole was shaken well and allowed to stand 20 minutes. The development of a rose colour indicated the presence of acetyl methyl carbinol. In the methyl red test a few drops of methyl red indicator were added to the remainder of the buffered dextrose culture. A red color indicated an acid pH, that is a positive M.R. test.

An organism being tested for its ability to produce catalase was inoculated onto a nutrient agar slant and incubated at 37° C for 24 hours. A few drops of fresh hydrogen peroxide solution were then placed on the culture. Effervescence from the bacterial colonies indicated the presence of catalase.

An organism being tested for its ability to produce nitrites from nitrates was inoculated into nitrate medium and incubated at 37° C for 24 hours. One ml. of each of Solution A and B was added. A red color indicated that nitrites were produced from nitrates.

An organism being tested for its ability to produce indole from tryptophane was inoculated into peptone broth and



incubated at 37° C for 24 hours. One ml. of ether was added to the culture and the whole shaken gently. Then one ml. of Ehrlich's reagent was introduced into the culture tube gently and allowed to collect between the medium and the ether. A pink color developing at the junction of the ether and the reagent indicated the presence of indole.



Experiments in which Bacteria were fed to Siphona irritans

To investigate the hypothesis that bacteria play a role in the development and continued existence of the horn fly the following experiments were carried out. Five bacterial cultures (Bacillus megatherium (two strains), Micrococcus epidermidis, Micrococcus flavus, and Micrococcus luteus) isolated from the interior of one lot of ten wild horn flies and one bacterial culture (Bacillus brevis) isolated from the combined intestinal tracts of two wild horn flies were selected for investigation. These were shipped to the Livestock Insect Laboratory at Lethbridge on nutrient agar slants in August, 1950. The surface of each slant was scraped with a sterile platinum loop and the scrapings suspended in each of six tubes containing ten milliliters of sterile distilled water and each culture was fed to one lot of flies. The laboratory reared flies (page 19), within twelve hours of each other in age, were placed in groups of 50 (25 males and 25 females), in seven lantern chimneys. Each test was run in duplicate so that in the 14 chimneys 700 flies were used. These flies were fed by the method described (page 21) but after the first day and for the following six days (this was as long as the ten ml. of bacterial suspension lasted) bacterial suspensions were fed in the mornings and the blood and sugar mixtures in the afternoons. In the seventh set, the controls, the flies received sterile distilled water only in the mornings and the blood and sugar mixture in the afternoons. Thereafter, all flies received a single daily meal of the



blood and sugar mixture until the end of the experiment. The flies in each set of chimneys received only one bacterial suspension. The bacterial suspensions, water, and blood and sugar mixture were offered to the flies soaked up in gauze pledges (page 20). On the twelfth day of the experiment some fresh cow manure was placed in each chimney and fresh manure renewed each day. On the thirteenth day of the experiment, eggs were deposited by flies receiving Micrococcus flavus, eight eggs were found in one chimney and one egg in the other. No eggs appeared in any of the other chimneys. By the time this experiment was completed it was too late in the season to obtain a sufficient number of flies of uniform age for a repeat experiment. The experiment was repeated in 1951 in order to confirm the positive result.

In August, 1951, three feeding experiments were started. The first an exact duplicate of the experiment which gave positive results in 1950; the second similar to the first except that no cow manure was placed in the test chimneys; in the third M. epidermidis was suspended in sterile nutrient broth. These four experiments were completed with negative results. After the sixth day of the experiment the ovaries of each dead female were examined but there was no evidence of significant growth and no eggs were laid. Two more feeding experiments were started in August, 1951, employing the six bacterial cultures used in the 1950 fly feeding experiments and two strains of M. epidermidis isolated in 1951. In the first experiment the cultures



were suspended in sterile distilled water and in the second in sterile nutrient broth. Both bacterial suspensions were prepared in concentrations higher than those used in the previous experiments in 1951. Results of these experiments were also negative. Experimental conditions were as close as possible to those of the original experiment but conditions did differ from the original in that the temperature conditions were not identical and the bacterial cultures used in the repeat experiments had been maintained on artificial media from the preceding summer.

The organisms tested do not appear to provide the growth factor or factors necessary for the development of the reproductive organs of the horn fly under laboratory conditions.



An experiment in which flies were fed on a calf

Concurrent with the feeding experiments employing bacterial cultures, attempts were made to obtain egg-laying by laboratory-reared flies fed on a calf. No one has reported feeding laboratory-reared Siphona on its normal host. Some difficulties were encountered in handling the flies but after a few abortive attempts the method finally adopted was as follows:

The flies were confined in lantern chimneys as in other feeding experiments. But the gauze covers were made secure by adhesive tape instead of elastic bands. This prevented the covers from coming off during the extra handling which the method entailed. A Holstein calf about three months old served as host. The hair was clipped from an area about six inches square on each side of the middle of the back of the calf. Two chimneys of flies were placed on the calf at each feeding. Just before each lantern chimney was placed on the calf the Petri plate base of the chimney was removed and the chimney placed on a thin metal disc about the same diameter as the Petri plate. The chimneys and discs were then placed on the clipped areas of the calf and held in position by a harness. After the chimneys were securely in place the metal discs were pulled out from under the chimneys. When the feeding period was over the metal discs were slipped carefully under the chimneys, the harness loosened and the chimneys removed. In this way the flies were fed for two



one-hour periods each day. An occasional fly escaped when the chimneys were transferred to and from the metal discs and Petri plates. At first the calf was tethered outside when flies were being fed but the heat of the sun was apparently too great, for most of the flies died while they were on the back of the calf. Thereafter the calf was tethered in the laboratory during the feeding periods.

In these tests the chimneys contained 25 to 50 flies each. The flies in two chimneys were fed on the calf while in two other chimneys the flies were fed only on citrated beef blood to serve as controls. Two experiments were carried to conclusion. In one of these the controls and the test flies when not on the back of the calf, were kept at room temperatures. In the other experiment they were kept at 25° C. No eggs were laid by the flies in these experiments but there was a pronounced increase in the amount of ovary development in the flies which were fed on the calf. The largest oocytes seen in some of the females were 0.50 ml. long and almost all of the females showed some development of ovaries.



A study of amino acid metabolism of the normal intestinal flora of *S. irritans* using paper partition chromatography

The amino acid metabolism of the bacteria comprising the normal bacterial flora of Siphona irritans was studied employing one dimensional paper partition chromatography. The same basic method of paper partition chromatography described by Consden, Gordon, and Martin (1944), with some modifications, was employed. A rectangular glass tank — one foot wide, three feet long, and two feet deep — was used. It had a rubber-stripped plate glass lid which was weighted down to maintain airtight conditions. Several inches from the top and running the length of the tank there were four glass rods. The two lower ones supported a glass trough 28 inches in length. The two other rods slightly to one side and above the first two were for the support of filter paper which passed over them from the trough. The filter paper was suspended vertically and held in position by a glass weight. Solvent used was that suggested by Proom and Woiwod (1949), 4n-butanol: 5 water: 1 glacial acetic acid (all parts V/V). After separation in a separatory funnel the top layer was put in the trough and the bottom layer in the bottom of the tank. Ninhydrin, 0.1% by W/V, (triketohydrindene hydrate) was added to the top layer and acted as a developer of amino acid spots in the chromatogram. Several days were required to bring the tank to equilibrium by saturation of the atmosphere inside the tank with the solvent. Standard size sheets of Whatman No. 1 filter paper



(22.5 x 18.25 inches) were marked in pencil with a series of dots each at a distance of three inches from the longer edge. These dots were placed one inch apart and labelled with an appropriate bacterial culture number. On each spot was placed five microliters of the material to be examined using a 0.2 ml. serological pipette. The sheet was then air dried.

The sheet was then suspended in the tank for one hour to saturate it with the atmosphere prevailing, before placing the edge, containing the material to be examined, into the solvent in the trough. After immersion in the solvent, the lid was put on, and the chromatogram was allowed to form by a downflow of the solvent — descending chromatography. After 36 hours the paper was removed from the tank and suspended in an oven at 80° C. for approximately one-half to one hour until dry. The oven contained a pan of steaming water; the steam quickened the appearance of the amino acid spots, intensified their color, and made them more permanent. Chromatograms were read with the aid of a "sheet-illuminator". This consisted of a wooden box, lined with white paper, and containing a source of light (a 60 watt, white light lamp). Sheets were placed over the open end and the light that passed through tended to make the spots more distinct. Organisms were grown in peptone water, nutrient broth, citrated sheep's whole blood, and in special media of known amino acid composition. Incubation was at 37° C for ten days. Five microliter aliquots were taken from each tube after 24, 48, and 72 hours, seven, and ten days incubation. The culture filtrates were examined by the technique described above.



PART III. MICROORGANISMS ISOLATED DURING THE  
PRESENT PROJECT AND THEIR METABOLIC BEHAVIOR



Fungi, Yeasts, and Molds associated with S. irritans

A tentative identification of fungi, yeasts, and molds associated with wild and laboratory reared horn flies was carried out. Out of twelve specimens of cattle faeces two contained the genus Asperigillus, three the genus Cladosporium, and one each the genera Geotrichum, Penicillium, and Oidium (or Oospora). From 66 swabs made from the skin sweat gland areas of cattle were isolated two organisms in the genus Trichothecium and one in the genus Thamnidium. Genus Asperigillus was isolated from the skin of a calf, from the area under a cage which was strapped to the animals back and which contained laboratory reared flies. From the external surfaces of seven lots comprising 72 wild flies one lot comprising five flies. harboured an organism in the genus Cladosporium. No other fungi were isolated from the external surfaces of wild flies. From nine lots comprising 117 treated and crushed wild flies, two lots comprising five flies each contained an organism in the genus Penicillium; one lot comprising 25 flies contained an organism with the morphology of an Actinomycete. From 28 lots comprising the intestinal tracts of 72 wild flies were isolated from each of six lots comprising 10 intestinal tracts the genus Asperigillus; from each of five lots comprising 13 intestinal tracts the genus Penicillium; from one intestinal tract the genus Hormodendrum; and from each of two tracts the genus Pullalaria. An organism in the order Phomales was isolated from one lot of 32 ova which were treated and crushed.



No fungi were isolated from the interior of larvae or pupae. An organism in the genus Pullalaria was isolated from one lot comprising five unfed laboratory reared flies.

Since fungi associated with horn flies did not appear consistently or in large numbers in cultures they were not studied further.



Bacteria associated with S. irritans

Criteria for bacterial identification are given below. In general they are based on the recommendations of Bergey's Manual of Determinative Bacteriology, Sixth Edition, 1948, and are confined to identification by morphological and biochemical characters. The organisms are listed alphabetically in order of their generic names.

Genus Achromobacter. Medium size rods of uniform shape. Gram negative to Gram variable. Peritrichous flagella or non-flagellate. Growth on nutrient agar was moderate, transparent, non-pigmented, and the colonies low convex. Litmus milk was unchanged. No acid or gas were produced from lactose, sucrose, maltose, or mannite. Activity on glucose was variable; one culture produced acid within 24 hours at 37° C. Gelatin was not liquefied. Urea was not decomposed within 48 hours at 37° C. On blood agar one culture produced true haemolysis in 24 hours.

Aerobacter aerogenes. Small size straight rods. Gram negative. Growth on nutrient agar was abundant, thick, white, moist, glistening, and spreading. Litmus milk was acidified and coagulated but not peptonized. Acid and gas were produced from lactose, glucose, sucrose, and mannite. Gelatin was not liquefied. Methyl red test was negative and acetyl methyl-carbinol was produced. Sodium citrate was used as a sole source of carbon. Hydrogen sulfide was not produced in Kligler's medium.



Aerobacter cloacae. Small size straight rods. Gram negative. Growth on nutrient agar was white, smooth, glistening, and spreading. Litmus milk was acidified, coagulated with gas production and was slowly peptonized. Acid and gas were produced from lactose, glucose, and sucrose. Gelatin was liquefied and glycerol not fermented. Methyl red test was negative and acetylmethylcarbinol was produced. Sodium citrate was used as a sole source of carbon. Hydrogen sulfide was not produced in Kligler's medium.

Genus Alcaligenes. Medium size rods of uniform shape. Gram negative. Peritrichous flagella or non-flagellate. Growth on nutrient agar slants was moderate, thin, transparent, and with a low dull margin. Nutrient broth was turbid. Litmus milk became alkaline and some strains produced a soft curd and peptonized the litmus milk. No acid or gas were produced from lactose, glucose, sucrose, or mannite. Acetylmethylcarbinol was not produced. Some cultures liquefied gelatin others did not. No odor was produced from nutrient broth. Urea was not decomposed within 48 hours at 37° C. No haemolysis was produced on blood agar.

Bacillus brevis. Medium to large size rods with endospores. Gram positive. Diameter of rods was less than 0.9 microns. Sporangia were distinctly bulged. Nutrient agar colonies were smooth, moist, spreading, and grey-white. Catalase positive. Sodium citrate was used as a sole source of carbon. Acid was produced from glucose and gelatin slowly liquefied. Genus Bacillus. Medium to large size rods with endospores.



Gram positive. Diameter of rods was variable. Growth on nutrient agar was rough, wrinkled, opaque, dull, and slightly spreading. All cultures were catalase positive.

Bacillus cereus var. mycoides. Medium size rods with endospores.

Gram positive. Diameter of rods more than 0.9 microns.

Growth on nutrient agar was thin, rhizoid, greyish, and spreading. Motile in nutrient broth. Catalase positive.

Acetyl methylcarbinol was produced. Acid produced from lactose.

Sodium citrate used as sole source of carbon. Gelatin was liquefied slowly.

Bacillus megatherium. Medium size rods with endospores.

Gram positive. Diameter of rods more than 0.9 microns.

Sporangia were not distinctly bulged. Nutrient agar colonies were large, soft, convex, entire, opaque, and creamy-white.

Catalase positive. Acetyl methylcarbinol was not produced.

Bacillus subtilis. Medium size rods with endospores. Gram positive. Diameter of the rods was less than 0.9 microns.

Sporangia not distinctly bulged. Growth on nutrient agar was abundant, rough, wrinkled, opaque, dull, and slightly spreading.

Catalase positive. Acetyl methylcarbinol was produced. Gelatin was liquefied. Nitrites were produced from nitrates.

Bacillus subtilis var. niger. Medium size rods with endospores.

Gram positive. Diameter of rods was less than 0.9 microns.

Sporangia not distinctly bulged. Growth on nutrient agar was rough, finely wrinkled, opaque, dull, slightly spreading, with a brownish tinge. Catalase positive. Acetyl methylcarbinol was produced. Gelatin was liquefied and acid produced from



arabinose with ammoniacal nitrogen. Black pigment was produced from tyrosin medium.

Escherichia coli. Short rods with rounded ends. Gram negative. Growth on nutrient agar was grey-white, moist, and homogeneous. Acid and gas were produced from lactose, glucose, and mannite. No acid or gas were produced from sucrose. Methyl red test was positive and no acetylmethylcarbinol was produced. Sodium citrate was not utilized as a sole source of carbon. Hydrogen sulfide was not produced.

Escherichia freundii. Short rods with rounded ends. Gram negative. Growth on nutrient agar was smooth, grey, shining, with a buttery consistency. Acid and gas were produced from lactose, glucose, and mannite but not from sucrose. Methyl red test was positive and no acetylmethylcarbinol was produced. Sodium citrate was utilized as a sole source of carbon. Hydrogen sulfide was produced.

Escherichia intermedium. Short rods with rounded ends. Gram negative. Growth on nutrient agar was smooth, white to grey in color, raised, and buttery in consistency. Acid and gas were produced from lactose, glucose, mannite, and sucrose. Methyl red test was positive and acetylmethylcarbinol was not produced. Sodium citrate was used as a sole source of carbon. Hydrogen sulfide was not produced.

Genus Flavobacterium. Medium size rods. Gram negative. Growth on nutrient agar was yellow to orange in color, shiny, convex, and non-spreading. No acid or gas were produced from lactose. Some forms produced acid from glucose. Acid was



produced from mannite and sucrose. Urea was not decomposed within 48 hours at 37° C.

Micrococcus aurantiacus. Spherical cells occurring singly and in clumps. Gram positive. Growth on nutrient agar was smooth, glistening, entire, and yellow to orange in color. Litmus milk was acidified and coagulated. Gelatin was liquefied. Nitrites were produced from nitrates. Ammonium phosphate was not utilized as a sole source of nitrogen. Blood was not haemolyzed.

Micrococcus candidus. Spheres less than one micron in diameter occurring singly. Gram positive. Growth on nutrient agar was moderate, transparent, cream in color, and glistening. Nitrites were not produced from nitrates. Ammonium phosphate was not utilized as a sole source of nitrogen. Litmus milk was unchanged. Gelatin was not liquefied. Acid was produced from glucose in 24 hours at 37° C. No acid or gas were produced from mannite. True haemolysis was produced on blood agar.

Micrococcus caseolyticus. Spheres less than one micron in diameter occurring in groups. Gram positive. Growth on nutrient agar was moderate and white in color. Nitrites were not produced from nitrates. Ammonium phosphate was utilized as a sole source of nitrogen. Litmus milk was acidified, coagulated, and peptonized. Gelatin was not liquefied. No acid or gas were produced from mannite.

Micrococcus conglomeratus. Spheres 0.8 microns in diameter occurring singly and in groups. Gram positive. Growth on nutrient agar was abundant, golden in color, moist, and opaque.



Nitrites were produced from nitrates. Ammonium phosphate was utilized as a sole source of nitrogen. Litmus milk was unchanged. Gelatin was liquefied slowly. No acid or gas were produced from mannite.

Micrococcus epidermidis. Spherical cells occurring singly and in clumps. Gram positive. Growth on nutrient agar was moderate, white, and opaque. Nitrites were produced from nitrates. Ammonium phosphate was not utilized as a sole source of nitrogen. Litmus milk was acidified, coagulated, and peptonized. Gelatin was liquefied. Acid was produced from glucose in 24 hours at 37° C. No haemolysis was produced on blood agar.

Micrococcus flayus. Spheres 0.9 microns in diameter occurring in groups. Gram positive. Growth on nutrient agar was scant, golden in color, and raised. Nitrites were not produced from nitrates. Ammonium phosphate was utilized as a sole source of nitrogen. Litmus milk was unchanged. Gelatin was not liquefied. Acid was produced from glucose and mannite. No haemolysis was produced on blood agar.

Micrococcus luteus. Spherical cells occurring singly and in groups. Gram positive. Growth on nutrient agar was scant, yellow in color, and raised. Nitrites were not produced from nitrates. Ammonium phosphate was utilized as a sole source of nitrogen. Litmus milk was unchanged. Gelatin was not liquefied. Acid was produced from mannite.

Genus Proteus. Straight rods. Gram negative. Growth on nutrient agar was dull, opaque, spreading. Litmus milk acidified



and coagulated. No acid was produced from lactose and sucrose. Acid and gas were produced from glucose and mannite. Urea was decomposed within 48 hours at 37° C. Most strains were actively motile at 25° C. but weakly so at 37° C.

Proteus rettgeri. Small rods. Gram negative. Growth on nutrient agar was moderate, greyish, opaque with even edges. No acid or gas were produced from lactose, sucrose, or maltose with the exception of one strain which produced acid from maltose. Acid was produced from glucose and mannite and slowly from glycerol. Acetylmethylcarbinol was not produced. Urea was decomposed within 48 hours at 37° C. Sodium citrate was used as a sole source of carbon. Hydrogen sulfide was not produced. Indole was formed.

Genus Streptococcus. Spherical cells in chains. Gram positive. Under anaerobic conditions growth on blood agar was scant, translucent, shiny, entire and greening haemolysis was produced in the surrounding medium.

Unidentified bacteria. By the process of elimination the following organisms have been excluded from the preceding genera and species:

Medium size aerobic rods. Gram positive. Non-sporing. Within the group colonial morphology was heterogeneous. No acid or gas were produced from lactose, glucose, or sucrose except for three cultures which produced variable fermentation reactions on these sugars. Their action on litmus milk and gelatin was variable. In nutrient broth at 30° C some were motile. The motile organisms possessed peritrichous flagella.



Medium size aerobic rods. Gram negative. Within the group colonial morphology was heterogeneous. Acid or acid and gas were produced from lactose, glucose, sucrose, and mannite slowly. Their action on litmus milk was variable.

Large size aerobic rods. Gram positive. Non-sporing. Within the group colonial morphology was heterogeneous. Lactose, glucose, and sucrose were not fermented except for four strains which produced acid from glucose and sucrose.

Anaerobic cocco-bacilli. Gram positive or Gram variable. Growth on nutrient agar was abundant, greyish-white, and raised. Acid and gas were produced from lactose, glucose, sucrose, and mannite. Nitrites were produced from nitrates. Methyl red test was positive and acetyl methyl carbinol was produced. Sodium citrate was not utilized as a sole source of carbon. Hydrogen sulfide was not produced. Gelatin was liquefied slowly.

Small size anaerobic rods. Gram negative. Within the group colonial morphology was heterogeneous. Acid was produced slowly from lactose but rapidly from glucose, sucrose, and mannite. Litmus milk was acidified and some cultures produced coagulation.

Medium size anaerobic rods. Gram positive. Non-sporing. Within the group colonial morphology was heterogeneous. Lactose was not fermented. One strain produced acid from glucose and sucrose. Action on litmus milk and gelatin was variable.

Large anaerobic rods. Gram positive. Non-sporing. Growth on nutrient agar was moderate and dry. Lactose, glucose,



sucrose, and maltose were not fermented. Litmus milk became alkaline slowly. Gelatin was liquefied slowly. Non-motile at 30° C.



Bacteria Isolated from the faeces of cattle

Sixty-five bacterial cultures were isolated from cattle faeces and perianal swabs. These included the genera Achromobacter, Alkaligenes, and Bacillus, *E. coli*, *E. intermedium*, *M. aurantiacus*, and three unidentified organisms (Table VI). A similar pattern of bacteria was obtained by Yanda (1952) in a study of 25 freshly passed cow droppings. Similar methods and materials were employed. This evidence tends to support the original findings. It seems unlikely that there will be much variation in faecal flora if one considers the digestive tract a true organ. Constancy characteristics, such as even temperature and pH, aid in maintaining a constant type of microbial population. It seems unlikely that bovine faeces form a large part of the food of the adult horn fly since it is only the female that is in contact with faeces and this is for a brief period only. According to a number of investigators, however, microorganisms present in faeces are good sources of vitamins (page 9). These vitamins may be available to the fly through ingested bacteria taken up during the developmental stages of the fly.



Table VI

## Bacteria Isolated from Cattle Faeces

genus species	Achromo- bacter	Alca- line	Bacil- lus	Escherichia	Flavo- bacterium	Micrococcus			Proteus	Unidentified
				coli	inter- medium	sauv- tciacus	caseo- lytic	U.S. flavus	aerobic	anae- robic
stools	7	2	5	6	2	2	1	4	1	1
swabs	1	3	0	0	0	0	2	1	0	0

Here the Arabic numerals indicate the number of times a particular genus or species was isolated from the specimens; for example E. coli was present in six of the twelve stool specimens examined.



Bacteria Isolated from the skin of cattle

One hundred ninety-eight cultures of bacteria were isolated from 66 swabs of the skin sweat gland areas of cattle. These included the same genera of bacteria as were isolated from cattle faeces but in addition Aerobacter aerogenes, Escherichia freundii, M. epidermidis, genus Proteus, and two unidentified organisms were isolated (Table VII). A comparison of Tables VI and VII shows an equivalent bacterial flora. A number of microorganisms must reach the skin from faeces because cattle often lie on soil contaminated with their own faeces. It is probable that the microbial flora of skin is less constant than that of faeces because the skin is exposed to seasonal and daily fluctuations in weather. Variation within the bacterial flora, however, is probably within comparatively narrow limits because some factors, such as temperature and food supply, are constant particularly next to the skin and in the deeper layers of sweat glands and hair follicles.

Six bacterial strains were isolated from the skin of a calf on which laboratory reared flies were fed. These included E. coli, E. intermedium and the genera Bacillus and Flavobacterium. In female horn flies feeding on this calf there was considerable development of ovaries on dissection.

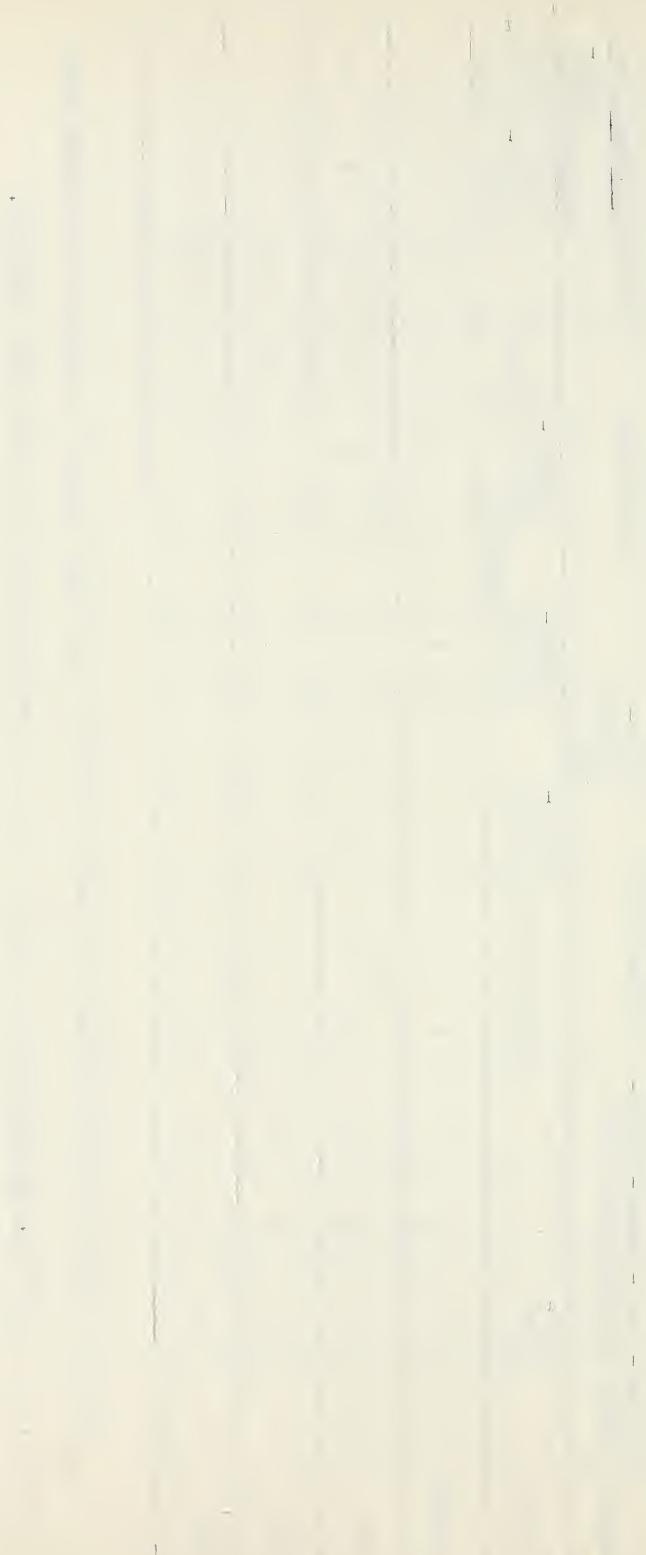


Table VII

Bacteria Isolated from Skin Sweat Gland Areas of Cattle.

Genera number of species examined	Achromo- bacter	Aero- bacter	Alcal- igines	Bacil- lus	Escherichia	Flavo- bacter- ium	Micrococcus	Proteus	Unidentified aerobic	Unidenti- fied anae- robic
	nero- genes			coli	freundii	inter- medium	buren- tacis	epider- midis	flavus	large Gram (-) rod
6 (22 swabs)	5	1	5	6	1	1	6	2	0	4
6 (22 swabs)	5	4	5	4	0	0	3	0	4	0
6 (22 swabs)	6	0	3	6	2	0	1	3	2	0
1 (1 swab)	0	0	0	1	1	1	0	0	0	0

Here the Arabic numerals indicate the number of times a particular genus or species was isolated from the cattle; for example M. epidermidis was isolated from the pastern area of the six cattle examined.



Bacteria isolated from the  
external surfaces of wild S. irritans

Fifty-seven bacterial cultures were isolated from the external surfaces of seven batches comprising 72 wild horn flies. Essentially the bacteria were the same as those isolated from the skin of cattle but did not include E. freundii, M. aurantiacus, M. flavus, and the genera Flavobacterium and Proteus; on the other hand, M. caseolyticus and an unidentified organism were isolated (Table VIII). Bacteria present on wild horn flies may have as their origin cattle faeces, having been "picked up" when the fly emerged or when the female deposited her eggs on cow manure, or from the skin of cattle where both male and female remain almost constantly. It is probable that any variation in the bacterial flora of the external surfaces of wild horn flies can be correlated with a change in skin flora of cattle.



Table VIII

Bacteria isolated from the external surfaces of wild *S. irritans*.

genera	Achromo- bacter	Alta- ligenes	Bacillus	Escherichia		Micrococcus		Unidentified
species				coli	inter- medium	candidus	caseo- lyticus	epidermidis
	2	3	5	1	1	2	1	2

Here the Arabic numerals indicate the number of times a genus or species has been isolated from seven lots of flies (comprising 72 flies).



Bacteria isolated from the interior of wild S. irritans

Of 20 lots comprising 243 treated and crushed wild flies, nine lots comprising 117 flies were found to contain internal organisms. Each lot contained from 1 to 25 flies. Sixty bacterial cultures were isolated. No organisms were isolated from the remaining 11 lots comprising 126 flies. In order of frequency of isolation the organisms were E. coli, from 4 lots (60 flies), P. rettgeri from 4 lots (100 flies), M. epidermidis from 3 lots (51 flies), B. subtilis from 3 lots (35 flies), E. intermedium from 1 lot (25 flies), genus Achromobacter from 1 lot (1 fly), genus Bacillus from 1 lot (1 fly) as shown in Table IX. These organisms must represent the bacteria of the digestive tract and trachea. Some of the above mentioned bacteria were also isolated from the digestive tract alone.



Table IX

Bacteria isolated from the interior of wild *S. irritans*

genus	Achromo-hacter	Escherichia		Bacillus	Micrococcus	Proteus
species		coli	inter-medium		subtilis	<i>epidermidis</i> <i>rettgeri</i>
	1	4	1	1	3	4

These bacteria were isolated from nine lots of flies.



Bacteria isolated from  
the intestinal tract of wild S. irritans

Of 57 lots comprising the dissected out intestinal tracts of 106 wild flies, 28 lots comprising 72 flies were found to contain organisms. Eighty-seven bacterial cultures were isolated and identified. No organisms were isolated from the remaining 29 lots comprising 34 intestinal tracts. In order of frequency the organisms were M. epidermidis from 11 lots (23 flies), genus Achromobacter from 10 lots (26 flies), M. candidus from 3 lots (7 flies), M. flavus from 2 lots (6 flies), genus Alcaligenes from 2 lots (10 flies), genus Bacillus from 2 lots (21 flies), B. brevis from 1 lot (2 flies), and 4 unidentified organisms from 3 lots (11 flies). In many instances more than one kind of organism was isolated from the same lot of flies. Each lot contained from 1 to 20 flies. The unidentified organisms in the genus Bacillus are thought to occur in the digestive tract adventitiously. Evidence to support this is found in 41 direct faecal smears examined. No organisms resembling spore bearers were seen. However, 37 of these smears showed either or both Gram negative and Gram positive cocci in large numbers. It has also been shown that the above mentioned organisms have a strong antagonistic action on the adventitious organisms in vitro. Observations also revealed that there was a mutual though weak antagonism among intestinal organisms themselves. It may well be that by this weak antagonistic action a



balance is maintained among organisms comprising the normal intestinal flora. It was found that certain of the organisms of the normal intestinal flora are capable of fermenting glucose, the blood sugar, within 24 hours at 37° C. The organisms were M. epidermidis, M. flavus, M. candidus, and an organism in the genus Achromobacter. The latter two organisms produced true haemolysis on blood agar, the Achromobacter organism markedly. In citrated whole blood M. epidermidis and M. flavus produced a soft clot in 8 hours at 37° C. Organisms comprising the normal intestinal flora may be of benefit to the fly by playing a role in the digestion of the fly.



Table X

Bacteria isolated from the intestinal tract of wild *S. irritans*.

genus	Achromo-	Alte-	Bacillus			Micrococcus			Unidentified		
	bacter	lignes			subtilis	candidus	flavus	epidermidis	aerobic	anaerobic	
species					var. niger				large Gram(-) rod	medium Gram(-) rod	
	11	3	2	1		3	3	12	3	1	



Bacteria isolated from the  
interior of ova, larvae, pupae, and unfed flies

Ova. Of 8 lots comprising 56 ova of wild horn flies, 2 lots comprising 33 ova were found to contain internal organisms when crushed. Four bacterial cultures were isolated. No organisms were isolated from the remaining lots. The organisms isolated were genus *Bacillus* from 1 lot (1 ovum), and two unidentified organisms from 1 lot (32 ova). The origin of microorganisms found inside intact eggs appears to be one not easily explained. In chickens if the egg is formed in an infected ovary, or passes through an infected oviduct, or if other parts of the egg are formed in infected organs, then it is not difficult to account for the presence of bacteria in newly laid eggs. Solowey et al (1947) showed that *Salmonella pullorum* could occur in eggs and food products made with these eggs and so cause outbreaks of the disease. In the horn fly it may be that a few bacteria enter the oviduct during copulation and so reach the inside of the egg with the entry of the sperm. After the egg has been laid on manure entry of the bacteria may occur through the micropyle. It is unlikely that microorganisms play an essential role in the maturation of horn fly eggs. If they were essential one would expect them to be isolated consistently from specimens; this has not been the case in this series. It may be concluded then that a few organisms occur in the ova adventitiously.



Larvae. Of 45 lots comprising 45 first instar larvae 42 lots contained organisms. No bacteria were isolated from three lots. The organisms were genus Achromobacter (from each of 22 larvae), genus Escherichia (from each of 12 larvae), genus Bacillus (from each of 7 larvae), and a medium size Gram negative rod (from 1 larva).

Of four lots comprising 8 third instar larvae, two lots comprising two larvae were found to contain organisms. Two bacterial cultures were isolated. No organisms were isolated from two lots. The organisms were E. freundii and E. intermedium. The feeding habits of larvae make it almost certain that bacteria will gain entry into their intestinal tracts. Glaser (1923) found a high incidence of bacteria in the larvae of the horn fly. He concluded that the larval stages of these flies are dependent upon certain accessory growth factors which must be ingested with the food. He also stated that microorganisms might be one of the principal sources of the accessory growth factors of some larval flies found breeding under certain conditions in nature, but did not go on to prove this statement.

Pupae. One strain of E. freundii was isolated from one lot of 50 crushed pupae. Glaser (1923) found that horn fly pupae contained very few bacteria and suggested that the bacteria are probably inhibited and destroyed as the insect transforms and assumes adult life. However, Leach (1933) found that bacteria survive in the lumen of the mid-intestine and to some extent in the cast-out linings of the fore- and hind-intestine



of the seed corn maggot, Hylemia cilicrura. It may be that bacteria survive in the gut of the horn fly by one or both of these methods during the development of the insect.

Unfed flies. Seventeen bacterial cultures were isolated from 24 unfed laboratory reared flies. The flies were in four lots. The organisms included A. aerogenes, A. cloacae, E. coli, E. intermedium, M. candidus, and M. epidermidis. From this it is evident that some bacteria are capable of survival in the fly through the developmental stages to the newly emerged fly. Some organisms present in the intestinal tract of wild flies may persist there from the larval and pupal stages. To some extent the variation in the flora of the intestinal tract of wild and unfed laboratory reared flies may be accounted for by the fact that the environment of each group of flies was different.



Bacteria isolated from  
laboratory reared flies fed M. flavus

From a preliminary feeding experiment three lots comprising eight laboratory reared flies fed M. flavus were found to contain internal organisms. Twenty-one bacterial cultures were isolated and identified. The organisms isolated were A. cloacae from two lots (7 flies), the genera Achromobacter, Bacillus, Streptococcus, and M. aurantiacus from one lot (three flies), B. cereus var. mycoides and two unidentified organisms (one Gram positive to Gram variable cocco-bacillus, and one medium size, non-sporing, Gram positive rod) from one lot (four flies), and M. epidermidis from one lot (one fly). From a duplicate experiment in which M. flavus was fed to nine lots comprising 39 flies one fly contained two Gram positive cocci, M. epidermidis and M. candidus. Forty-four bacterial cultures were isolated, however, Gram positive cocci were the only organisms identified, the majority of the remaining organisms being small Gram negative rods. In neither experiment was there any evidence of the persistence of M. flavus. It may be that this organism is digested by the insect.



Amino acid metabolism of bacteria

comprising the normal intestinal flora of the horn fly

Utilization or production of an amino acid was indicated by the diminution or increase, respectively, in intensity of its chromatographic "spot" in comparison with a control. Production of a polypeptide was indicated by the appearance of a new "spot", in the shape of an inverted "V", in comparison with a control. In this way the amino acid metabolism of the normal intestinal flora of Siphona (page 83) was studied in simple media of complex amino acid composition, and in special media of known amino acid composition.

Amino acid metabolism in media of complex protein composition.

Tryptophane assay medium (Difco) plus tryptophane was used at the beginning of experimental work because it contained amino acids which could be easily separated and identified by paper partition chromatography. Similar results were obtained with media such as peptone water, nutrient broth, and citrated whole blood.

M. epidermidis. One strain was examined. From Table XI it may be seen that the basic group of amino acids was not utilized in any of the media except whole blood. Aspartic acid was utilized extensively in all media except whole blood where it was used sparsely. Serine and glycine were utilized in all media to a variable degree. Glutamic acid was utilized in all media except Medium I which was unchanged. The amino acids threonine and alanine were metabolized as follows; they



were unchanged in Medium I; threonine was sparsely utilized in peptone water but later a large amount of it and a small amount of alanine were produced; both threonine and alanine were used extensively in nutrient broth. Threonine, alanine, methionine, valine, phenylalanine, isoleucine, and leucine did not appear in filtrates of blood examined chromatographically. A small amount of methionine was produced in peptone water but was later utilized. None of the other amino acids contained in the media was visibly metabolized.

Genus Achromobacter. Four cultures were examined. Selection was on the basis of different biochemical activity of each strain. From Table XII it may be seen that the basic group of amino acids was not visibly metabolized except in Medium I where it was utilized sparsely. Aspartic acid and serine were utilized and/or produced depending on the bacterial culture. Serine in whole blood was unchanged by all cultures. Glycine was utilized to a variable degree by one culture (B125b) except in blood where it was unchanged. In whole blood incubated for 24 hours this culture produced a "polypeptide" between glycine and glutamic acid on the chromatogram but was utilized in 48 hours and did not appear again after this time. Glutamic acid was, utilized sparsely by this culture in all media, and produced by two cultures in whole blood. Threonine, alanine, methionine, and valine were metabolized to a slight degree depending on the culture. Phenylalanine, isoleucine, and leucine were not visibly metabolized.



M. candidus. One culture was examined From Table XIII it may be seen that in all the media tested the basic group of amino acids was not visibly metabolized. Aspartic acid was produced in all the media except whole blood. In blood it was not visibly metabolized and in peptone water aspartic acid was utilized. Serine was sparsely used in Medium I and unchanged in the remainder. Glycine and alanine were not visibly metabolized. Glutamic acid was used sparsely in nutrient broth and a small amount was produced in whole blood but later utilized. Threonine was utilized sparsely in peptone water. Methionine was utilized sparsely in peptone water and valine sparsely in nutrient broth.

M. flavus. One culture was examined. From Table XIV it may be seen that the basic group of amino acids was not visibly metabolized except in whole blood where it was utilized to a large degree. Aspartic acid and serine were utilized extensively and glycine utilized to a lesser degree. Glutamic acid was utilized sparsely in Medium I, was produced to a small degree in peptone water but was later utilized to a large degree, and was utilized to a large degree in nutrient broth and whole blood. Threonine and alanine were metabolized variably. Methionine, valine, phenylalanine, leucine, and isoleucine were not visibly metabolized.

Genus Alcaligenes. One culture was examined. While genus Alcaligenes was not isolated from the horn fly with the same frequency as other intestinal bacteria it is included in these amino acid studies because of its close taxonomic relationship



to genus Achromobacter. From Table XV it may be seen that the basic group of amino acids was utilized sparsely in Medium I, and not visibly metabolized in the remaining media. In the media tested aspartic acid and serine were metabolized variably or not at all. Glutamic acid, was utilized in Medium I and in peptone water, was not visibly metabolized in nutrient broth, and was produced in considerable amounts from whole blood. Alanine, methionine, isoleucine, and leucine were metabolized sparsely. Phenylalanine was not visibly metabolized.

Of the four media used, metabolism seemed most extensive in nutrient broth, peptone water, and whole blood. Growth in Medium I (Tryptophane assay medium plus tryptophane) was somewhat retarded.

Amino Acid Metabolism in Media of known Composition. The purpose of this portion of the investigation is to gain additional information regarding the amino acid metabolism of the normal intestinal bacteria of the horn fly. The medium (Medium III) in which the Micrococci were grown is based on a special medium devised by Fildes and Richardson (1937) devised for the cultivation of Staphylococci. The Gram negative small rods, namely the genera Achromobacter and Alcaligenes, were grown in a special medium (Medium II) found by Yanda (1952) to contain essential amino acids for supporting the growth of certain Gram negative rods (Paracolobactrum).

M. epidermidis. From Table XVI it may be seen that aspartic acid was utilized sparsely but was later produced to a small



degree. Methionine was produced in considerable amount but was later utilized. The remaining amino acids were not visibly metabolized.

Genus Achromobacter. Two cultures were examined. From Table XVII it may be seen that both cultures produced aspartic acid to a variable degree but that the aspartic acid was later utilized by both. The remaining amino acids were not visibly metabolized.

M. candidus. From Table XVI it may be seen that aspartic acid was produced first, utilized later, and finally produced again. Methionine was produced but later utilized. The remaining amino acids were not visibly metabolized.

M. flavus. From Table XVI it may be seen that aspartic acid was utilized sparsely. Methionine was produced. The remaining amino acids were not visibly metabolized.

Genus Alcaligenes. One culture was examined. From Table XVII it may be seen that aspartic acid was produced but was later utilized. The remaining amino acids were not visibly metabolized.

Amino acid metabolism in media containing certain amino acids was not so extensive as that in complex amino acid media. The pattern of amino acid metabolism also varied in some instances.



Table XI

Amino acid metabolism of M. epidermidis in complex protein media.

Medium	Time Incubated	Amino acid metabolized										
		B	As	S	G	GA	T	A	M	V	P	L
I	24 hrs.	-	XX	-	-	-	-	-	-	-	-	-
	48 hrs.	-	XX	-	-	-	-	-	-	-	-	-
	72 hrs.	-	XX	-	-	-	-	-	-	-	-	-
	10 days	-	XX	X	X	-	-	-	-	-	-	-
Peptone Water	24 hrs.	-	X	-	-	-	-	-	-	-	-	-
	48 hrs.	-	XX	-	X	-	-	-	+	-	-	-
	72 hrs.	-	X	-	-	-	-	-	-	-	-	-
	7 days	-	X	-	X	-	X	-	-	-	-	-
	10 days	-	XX	X	XX	XX	++	+	-	-	-	-
Nutrient Broth	24 hrs.	-	XX	-	-	X	-	-	-	-	-	-
	48 hrs.	-	X	-	-	-	-	-	-	-	-	-
	72 hrs.	-	XX	XX	XX	-	XX	-	-	-	-	-
	7 days	-	XX	XX	XX	XX	XX	XX	-	-	-	-
	10 days	-	XX	XX	XX	XX	XX	XX	-	-	-	-
Citrated Whole Blood	24 hrs.	X	-	X	XX	XX						
	48 hrs.	XX	-	XX	XX	XX						
	72 hrs.	XX	X	XX	XX	XX						

Amino Acids

- B - basics (lysine, arginine, histidine)
- As - aspartic acid
- S - serine
- G - glycine
- GA - glutamic acid
- T - threonine
- A - alanine
- M - methionine
- V - valine
- P - phenylalanine
- L - isoleucine, leucine

Symbols

- XX - complete or appreciable utilization
- X - slight utilization
- - not metabolized
- +- slight production
- ++ - appreciable production

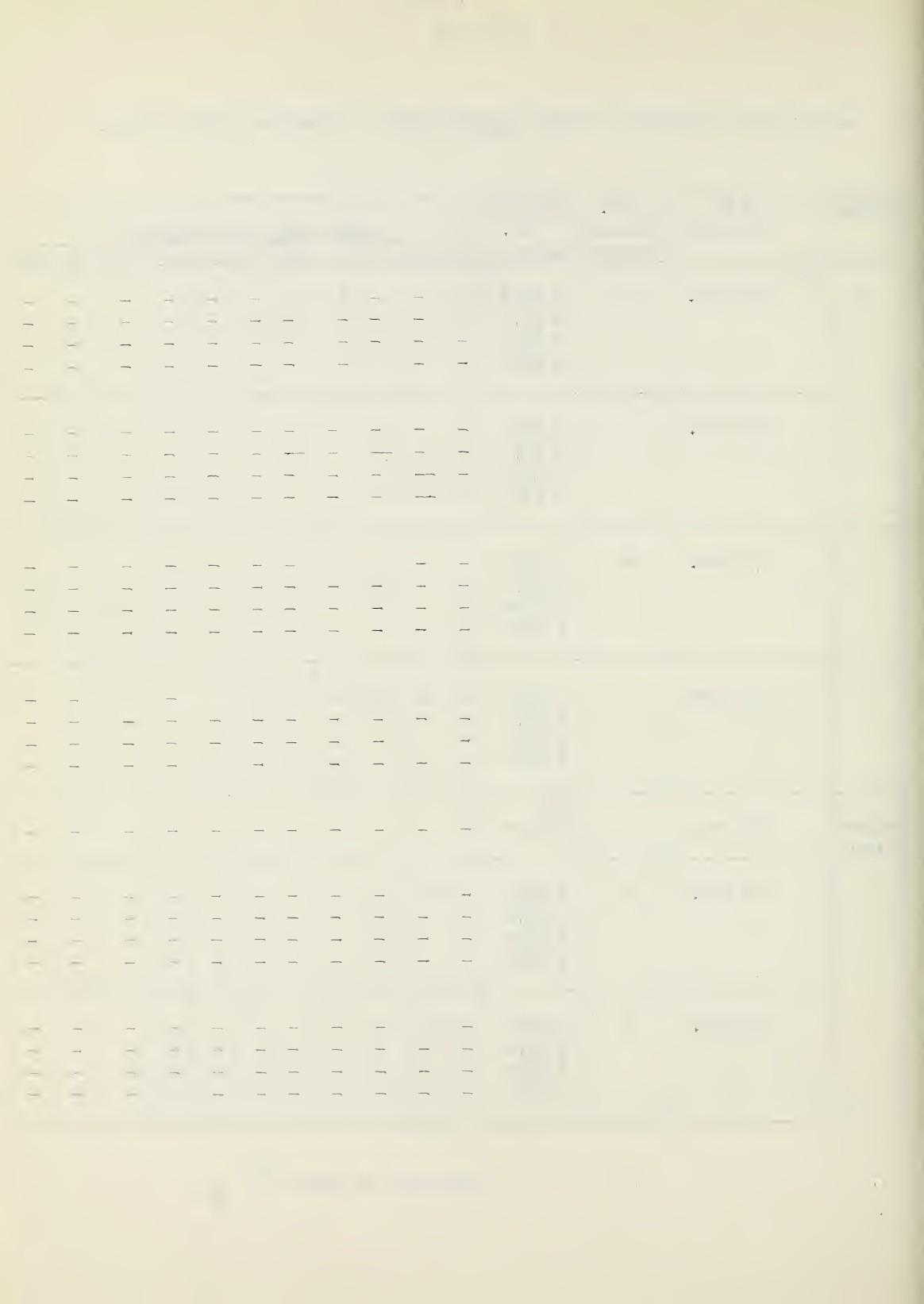
These abbreviations and symbols have the same connotation for Tables XI to XVII.



Table XII

Amino acid metabolism of genus Achromobacter in complex protein media.\*

Medium	Time Incubated	No. Cultures Tested	Culture No. Reacting	Amino acids metabolized										
				B	As	S	G	GA	T	A	M	V	P	L
I	24 hrs.	4	B 125b	x	-	-	x	x	-	-	-	-	-	-
			B 127c	x	-	-	-	-	-	-	-	-	-	-
			B 132a	-	-	-	-	-	-	-	-	-	-	-
			B 135b	-	+	x	-	-	-	-	-	-	-	-
	48 hrs.	4	B 125b	-	-	-	-	-	-	-	-	-	-	-
			B 127c	-	+	++	-	-	++	-	-	-	-	-
			B 132a	-	++	-	-	-	-	-	-	-	-	-
			B 135b	x	++	-	-	-	-	-	-	-	-	-
	72 hrs.	4	B 125b	-	+	x	x	-	-	-	-	-	-	-
			B 127c	-	+	-	-	-	-	-	-	-	-	-
			B 132a	-	-	-	-	-	-	-	-	-	-	-
			B 135b	-	-	-	-	-	-	-	-	-	-	-
	10 days	4	B 125b	x	xx	xx	xx	x	x	x	-	x	-	-
			B 127c	-	+	-	-	-	-	-	-	-	-	-
			B 132a	-	x	-	-	-	-	-	-	-	-	-
			B 135b	-	+	-	-	x	-	x	-	-	-	-
Peptone Water	24 hrs	4	All Cultures	-	-	-	-	-	-	-	-	-	-	-
	48 hrs.	4	B 125b	-	xx	-	-	-	-	-	+	-	-	-
			B 127c	-	-	-	-	-	-	-	-	-	-	-
			B 132a	-	-	-	-	-	-	-	-	-	-	-
			B 135b	-	-	-	-	-	-	-	-	-	-	-
	72 hrs.	4	B 125b	-	xx	-	-	-	-	-	-	-	-	-
			B 127a	-	-	-	-	-	-	-	-	-	-	-
			B 132a	-	-	-	-	-	-	-	-	-	-	-
			B 135b	-	-	-	-	-	-	-	x	-	-	-



Amino acid metabolism of genus Achromobacter in complex protein media  
(cont'd.)

Medium	Time Incubated	No. Cultures Tested	Culture No. Reacting	Amino acids metabolized												
				B	As	S	G	GA	T	A	M	V	P	L		
Peptone Water	7 days	4	B 125b	-	XX	X	XX	X	X	XX	--	--	--	--	--	
			B 127c	-	++	-	-	-	-	-	-	-	-	-	-	
			B 132a	-	++	-	-	-	-	-	-	-	-	-	-	
			B 135b	-	++	-	--	-	-	-	X	-	-	-	-	
	10 days		B 125b	-	XX	-	XX	X	X	XX	-	-	-	-	-	
			B 127c	-	-	-	-	-	-	-	-	-	-	-	-	
			B 132a	-	-	-	-	-	-	-	-	-	-	-	-	
			B 135b	-	-	-	--	-	-	-	-	-	-	-	-	
Nutrient Broth	24 hrs.	4	B 125b	-	-	-	-	-	-	-	-	-	-	-	-	
			B 127c	-	-	-	-	-	-	-	-	-	-	-	-	
			B 132a	-	-	-	-	-	-	-	-	-	-	-	-	
			B 135b	-	-	X	X	X	-	-	-	-	-	-	-	
	48 hrs.	4	B 125b	-	XX	X	X	-	-	X	-	-	-	-	-	
			B 127c	-	-	-	-	-	-	-	-	-	-	-	-	
			B 132a	-	-	-	-	-	-	-	-	-	-	-	-	
			B 135b	-	-	-	-	-	-	-	-	-	-	-	-	
	72 hrs.	4	B 125b	-	XX	-	X	-	-	X	-	-	-	-	-	
			B 127c	-	+	-	-	-	-	-	-	-	-	-	-	
			B 132a	-	+	-	-	-	-	-	-	-	-	-	-	
			B 135b	-	+	-	-	-	-	-	-	-	-	-	-	
	7 days	4	B 125b	-	XX	X	X	-	-	X	-	-	-	-	-	
			B 127c	-	-	-	-	-	-	-	-	-	-	-	-	
			B 132a	-	-	-	-	-	-	-	-	-	-	-	-	
			B 135b	-	-	-	-	-	-	-	-	-	-	-	-	
	10 days	4	B 125b	-	XX	X	X	-	-	XX	-	-	-	-	-	
			B 127c	-	-	-	-	-	-	-	-	-	-	-	-	
			B 132a	-	-	+	-	-	-	-	-	-	-	-	-	
			B 135b	-	-	-	X	-	-	-	-	-	-	-	-	

continued on page 98



Table XII

Amino acid metabolism of genus Achromobacter in complex protein media  
(cont'd.)

Medium	Time Incubated	No. Cultures Tested	Culture No. Reacting	Amino acids metabolized										
				B	As	S	G	GA	T	A	M	V	P	L
Citrated Whole Blood	24 hrs.	4	B 125b	-	++	-	-	-	↔	-				
			B 127c	-	++	-	-	-	-	-				
			B 132a	-	-	-	-	-	-	-				
			B 135b	-	-	-	-	-	-	-				
	48 hrs.	4	B 125b	-	XX	-	-	-	-	-				
			B 127c	-	XX	-	-	-	-	++				
			B 132a	-	-	-	-	-	-	+				
			B 135b	-	-	-	-	-	-	+				
	72 hrs.	4	B 125b	-	-	-	-	-	XX					
			B 127c	-	XX	-	-	-	-	++				
			B 132a	-	XX	-	-	-	-	-				
			B 135b	-	XX	-	-	-	-	-				

\*PP = polypeptide formation. The biochemical composition of this polypeptide was not investigated.



Table XIII

## Amino acid metabolism of *M. candidus* in complex protein media

Medium	Time Incubated	Amino acid metabolized										
		B	As	S	G	GA	T	A	M	V	P	L
I	24 hrs.	-	+	X	--	--	--	--	--	--	--	--
	48 hrs.	-	+	-	-	-	-	-	-	-	-	-
	72 hrs.	-	+	-	-	-	-	-	-	-	-	-
	10 days	-	++	-	-	-	-	-	-	-	-	-
Peptone Water	24 hrs.	-	-	-	-	-	-	-	-	-	-	-
	48 hrs.	-	-	-	-	-	-	-	-	-	-	-
	72 hrs.	-	++	-	-	-	-	-	-	X	-	-
	7 days	-	++	-	-	-	-	X	-	-	-	-
	10 days	-	XX	-	-	-	X	-	-	-	-	-
Nutrient Broth	24 hrs.	-	-	-	-	-	-	-	-	-	-	-
	48 hrs.	-	-	-	-	-	-	-	-	-	X	-
	72 hrs.	-	+	-	-	-	-	-	-	-	-	-
	7 days	-	-	-	-	-	-	-	-	-	-	-
	10 days	-	-	-	-	X	-	-	-	-	-	-
Citrated Whole Blood	24 hrs.	-	-	-	-	-						
	48 hrs.	-	-	-	-	-	+					
	72 hrs.	-	-	-	-	-	-					



Table XIV

Amino acid metabolism of M. flavus in complex protein media.

Medium	Time Incubated	Amino acid metabolized											
		B	As	S	G	GA	T	A	M	V	P	L	
I	24 hrs.	-	+	x	-	-	x	x	-	-	-	-	
	48 hrs.	-	-	-	x	x	-	-	-	-	-	-	
	72 hrs.	-	xx	-	-	-	-	-	-	-	-	-	
	10 days	-	xx	x	x	-	-	-	-	-	-	-	
Peptone Water	24 hrs.	-	xx	-	-	-	-	-	-	-	-	-	
	48 hrs.	-	xx	x	-	-	-	-	-	-	-	-	
	72 hrs.	-	x	x	-	-	-	-	-	-	-	-	
	7 days	-	x	x	x	+	x	-	-	-	-	-	
	10 days	-	xx	x	xx	xx	++	+	-	-	-	-	
Nutrient Broth	24 hrs.	-	xx	x	-	-	x	-	-	-	-	-	
	48 hrs.	-	x	-	x	x	x	x	-	-	-	-	
	72 hrs.	-	xx	xx	xx	-	xx	-	-	-	-	-	
	7 days	-	xx	-	-	-							
	10 days	-	xx	-	-	-							
Citrated Whole Blood	24 hrs.	-	x	-	x	xx							
	48 hrs.	xx	-	xx	xx	xx							
	72 hrs.	xx	x	xx	xx	xx							

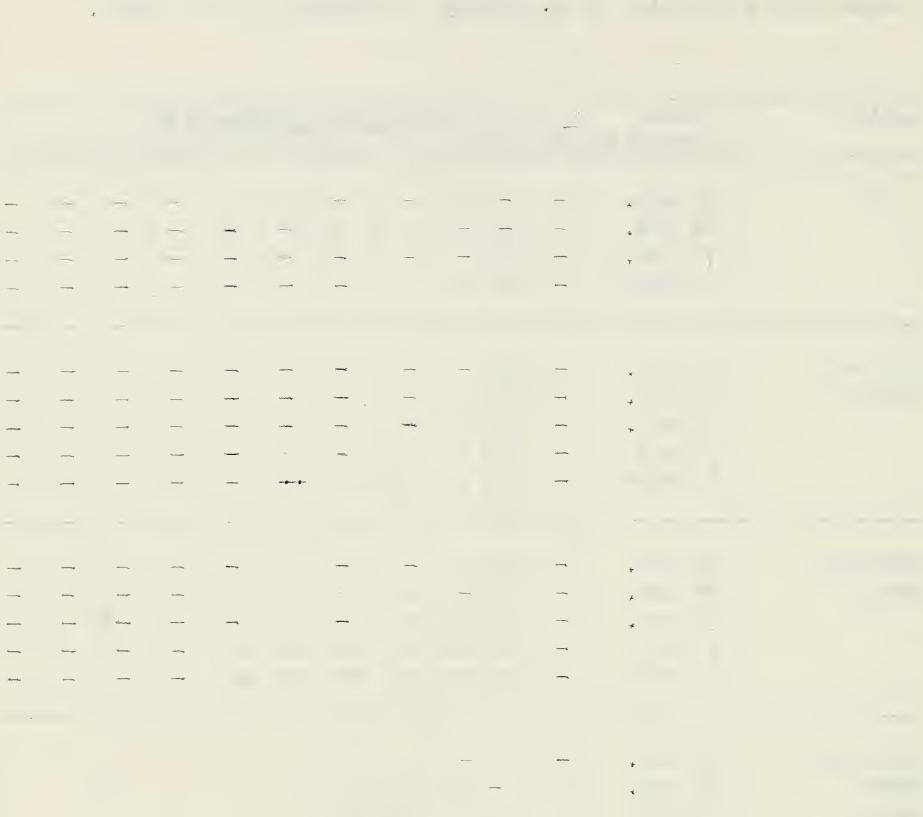


Table XV

Amino acid metabolism of genus Alcaligenes in complex protein media

Medium	Time Incubated	Amino acid metabolized										
		B	As	S	G	GA	T	A	M	V	P	L
I	24 hrs.	-	+	-	-	-	-	-	-	-	-	-
	48 hrs.	-	+	+	-	-	-	-	-	+	-	+
	72 hrs.	-	XX	-	-	-	-	-	-	-	-	-
	10 days	X	XX	X	-	X	-	X	-	-	-	-
Peptone Water	24 hrs.	-	X	-	-	-	-	-	-	-	-	-
	48 hrs.	-	-	-	-	-	-	-	-	-	-	-
	72 hrs.	-	++	-	-	-	-	-	-	-	-	-
	7 days	-	-	-	-	-	X	X	-	-	-	-
	10 days	-	-	-	-	-	-	-	-	-	-	-
Nutrient Broth	24 hrs.	-	-	-	-	-	-	-	-	-	-	-
	48 hrs.	-	-	-	-	-	-	-	-	-	-	-
	72 hrs.	-	+	X	X	-	-	-	-	-	-	-
	7 days	-	-	-	-	-	-	-	-	-	-	-
	10 days	-	X	-	-	-	-	-	-	-	-	-
Citrated Whole Blood	24 hrs.	-	-	-	-	-	-	-	-	-	-	-
	48 hrs.	-	-	-	++	++	-	-	-	-	-	-
	72 hrs.	-	-	-	XX	++	-	-	-	-	-	-



Table XVI

Amino acid metabolism of Micrococcii in Medium III

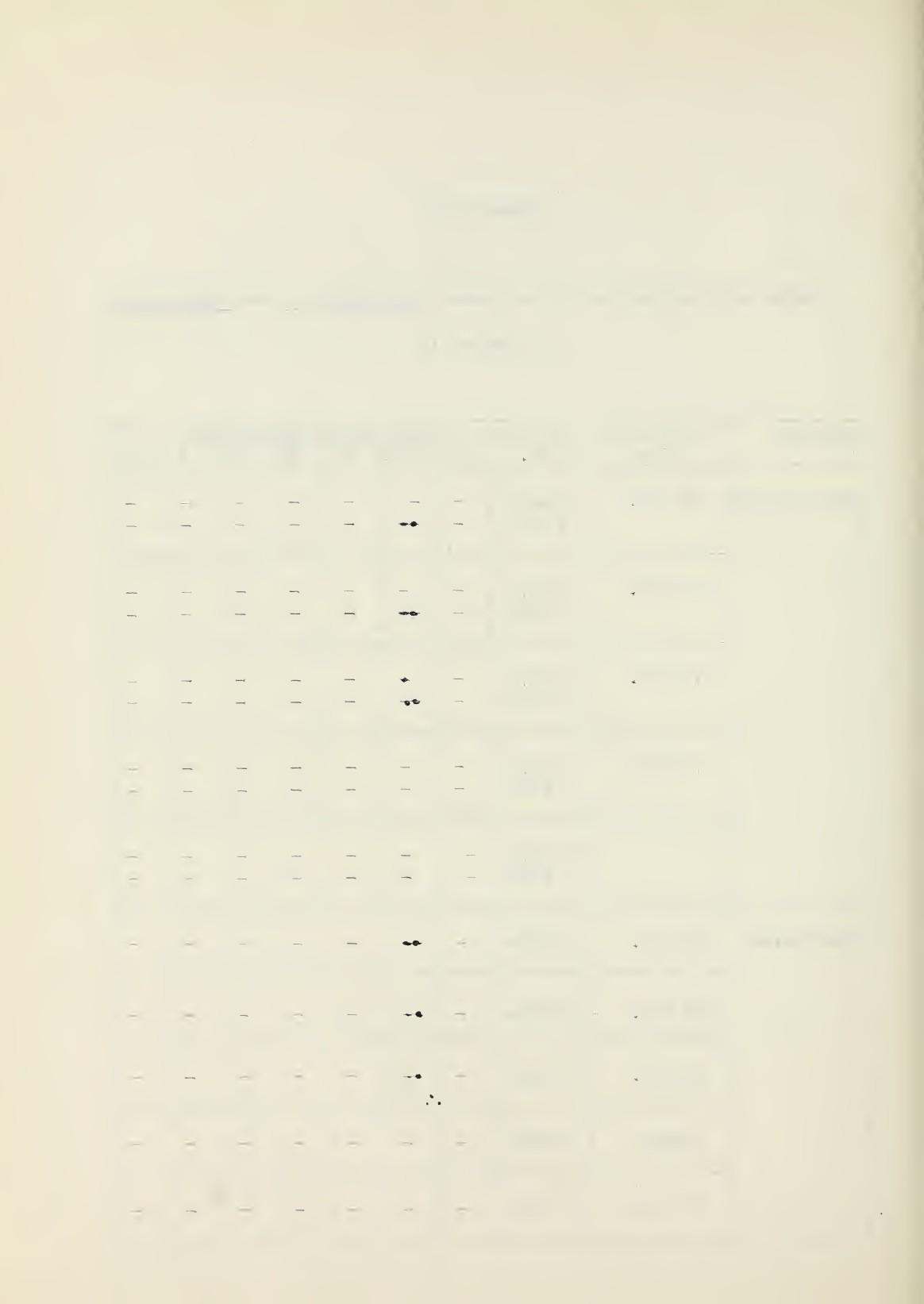
Organism	Time Incubated	Amino acid metabolized										
		B	As	S	G	GA	T	A	M	V	P	L
<i>M. epidermidis</i>	24 hrs.	-	-	-	-	-	-	-	+	-	-	-
	48 hrs.	-	X	-	-	-	-	-	+	-	-	-
	72 hrs.	-	-	-	-	-	-	-	++	-	-	-
	7 days	-	-	-	-	-	-	-	++	-	-	-
	10 days	-	+	-	-	-	-	-	-	-	-	-
<i>M. candidus</i>	24 hrs.	-	++	-	-	-	-	-	-	-	-	-
	48 hrs.	-	X	-	-	-	-	-	+	-	-	-
	72 hrs.	-	-	-	-	-	-	-	X	-	-	-
	7 days	-	-	-	-	-	-	-	X	-	-	-
	10 days	-	+	-	-	-	-	-	-	-	-	-
<i>M. flavus</i>	24 hrs.	-	-	-	-	-	-	-	+	-	-	-
	48 hrs.	-	X	-	-	-	-	-	+	-	-	-
	72 hrs.	-	-	-	-	-	-	-	+	-	-	-
	7 days	-	-	-	-	-	-	-	++	-	-	-
	10 days	XX	+	X	X	-	-	-	-	-	-	-



Table XVII

Amino acid metabolism of the genera Achromobacter and Alkaligenes  
in Medium II

Organism	Incubation Time	Culture No.	Amino Acids Metabolized						
			B	As	S	GA	A	V	L
Achromobacter	24 hrs.	B 127c	-	-	-	-	-	-	-
		B 135b	-	++	-	-	-	-	-
	48 hrs.	B 127c	-	-	-	-	-	-	-
		B 135b	-	++	-	-	-	-	-
	72 hrs.	B 127c	-	+	-	-	-	-	-
		B 135b	-	++	-	-	-	-	-
Alkaligenes	7 days	B 127c	-	-	-	-	-	-	-
		B 135b	-	-	-	-	-	-	-
		B 127c	-	-	-	-	-	-	-
		B 135b	-	-	-	-	-	-	-
	10 days	B 163a	-	-	-	-	-	-	-



PART IV. SUMMARY AND CONCLUSIONS



Summary

Because no evidence has been accumulated in favor of the original hypothesis a discussion of the role of bacteria in the continued existence of S. irritans is omitted. The present thesis is a systematic account of microorganisms and particularly bacteria associated with wild and laboratory reared horn flies. Work was carried out over a two year period and organisms were isolated from the external surfaces of wild flies, from their crushed bodies after the external surfaces had been disinfected, and from their intestinal tracts after dissection. Limited studies were made of ova, larvae, and pupae of wild horn flies and of the skin and faeces of cattle. Organisms isolated from cattle fell into a pattern similar to that isolated from wild horn flies. Microorganisms were also isolated from fed and unfed laboratory reared flies. The inevitable conclusion is that the bacterial flora of the horn fly is derived from its environment and directly related to it.

During this project several interesting observations were made. Although in general the intestinal bacteria of the horn fly appeared representative of its environment yet they were obviously influenced by some unknown endogenous factor. Early season flies were found to contain mainly Gram positive cocci whereas in late season flies small Gram negative rods predominated. Although some degree of mutual antagonism has been shown to exist in strains of intestinal



bacteria the demonstrated change in bacterial population cannot be ascribed to this. Nevertheless this antagonism is probably associated with the relative proportions of individual strains at any one time.

A study of the nitrogen metabolism of bacteria derived from the intestine of the horn fly showed that certain strains were capable of synthesizing a number of amino acids. This raises a new hypothesis, that such bacteria, although apparently not related to the fertility factor, may yet play a vital role in the insect's metabolism.

Much work has been done on the elaboration of techniques necessary to handling, rearing, sterilizing, and dissecting horn flies and these methods have been described in detail. It is hoped that later workers who may wish to pursue further investigations in this practically unexplored field may be saved much time and trouble by consulting these descriptions.

#### Conclusions

1. The fertility of *Siphona irritans* is not determined by any factor directly derived from its intestinal or environmental bacteria.
2. The microorganisms associated with wild *S. irritans* are represented by a relatively small number of genera.
3. The normal intestinal bacteria of *S. irritans* are represented by the genera *Achromobacter*, *Alcaligenes*, and *Micrococcus*.
4. The normal intestinal bacteria of *S. irritans* exert a mutual but mild antagonism.



5. Internally, ova harbour a few adventitious bacteria.
6. Bacteria are capable of surviving in the gut during the developmental stages from larvae to newly emerged flies.
7. The bacteria on the external surfaces of S. irritans fall into a pattern similar to the bacteria on the skin and in the faeces of cattle, that is, the bacteria of the fly's natural environment.
8. Some of the normal intestinal bacteria are capable of synthesizing certain amino acids. These may play some part in the nutrition of the insect.



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APPENDIX



Appendix

Nutrient Broth

Peptone (Difco)	1.0 gm.
NaCl	0.5
Beef extract (Difco)	0.5
Distilled water	100.0 ml.

Adjust the pH to 7.6 with N NaOH. This and all subsequent media were autoclaved at 121.5° C at 15 pounds pressure for 20 minutes unless otherwise mentioned.

Nutrient Agar

Nutrient broth	1.0 l.
Agar (Difco)	20.0 gm.

Blood Agar

Nutrient agar	1.0 l.
Sheep's blood (citrated)	50.0 ml.

Blood was added aseptically to sterile nutrient agar.

Alpha-naphthol Solution

Ethyl alcohol (absolute)	1.0 l.
Alpha-naphthol	5.0 gm.



Ammonium phosphate Agar

Agar (Difco)	1.5 gm.
Ammonium phosphate	0.1
Glucose	1.0
Potassium chloride	0.02
Magnesium sulphate	0.02
Brom-cresol purple (1%)	1.0 ml.
Distilled water	100.0 ml.

Buffered Glucose Broth

Peptone (Difco)	5.0 gm.
Glucose	5.0
K <sub>2</sub> HPO <sub>4</sub>	5.0
Distilled water	800.0 ml.

Sterilization without pressure for three successive days.

Carnoy's Fixative

Ethyl alcohol (absolute)	60 ml.
Chloroform	30
Acetic acid (glacial)	10

Citrate Agar

Ammonium sulphate	1.0 gm
Magnesium sulphate	0.2
Dipotassium phosphate	1.0
Sodium citrate	3.0

continued on page 118



Citrate Agar (cont'd.)

Sodium chloride	5.0 gm.
Agar (Difco)	20.0
Phenol red	0.012
Distilled water	1.0 l.
Adjust pH to 7.0.	

Gelatin Medium

Gelatin	50.0 gm.
Peptone (Difco)	10.0
Disodium phosphate	2.0
Glucose	1.0
Sodium thioglycollate	1.0
Distilled water	1.0 l.

Glycerol Broth

1% glycerol in nutrient broth

1% Andrades indicator

5 ml. in culture tube containing inverted Durham tube.

Ehrlich's Reagent

Para-dimethyl-amino-benzaldehyde	5 gm.
Butyl alcohol	75 ml.
HCl (concentrated)	25 ml.



Kligler's Iron Medium (Difco)

Beef Extract	3.0 gm.
Yeast	3.0
Peptone	15.0
Proteose peptone	5.0
Lactose	10.0
Glucose	1.0
Ferrous sulphate	0.2
Sodium chloride	5.0
Sodium thiosuophate	0.3
Agar	2.0
Phenol red	0.024

Fifty-five grams of this material was dissolved in one liter of distilled water, tubed in 5 ml. amounts and autoclaved.

Littman's Oxgall Agar (Difco)

Peptone	10.0 gm.
Glucose	10.0
Oxgall	15.0
Agar	20.0
Crystal violet	0.01

Fifty-five grams of this material and 30 units of Merck's Streptomycin (calcium chloride complex) were dissolved in one liter of distilled water and autoclaved.



Litmus milk

Skimmed milk 100.0 ml.

Brom-cresol purple (1%) 1.0

Sterilization without pressure for one hour on  
three successive days.

Loeffler's Serum Slants

Human Serum 450 ml.

Nutrient broth 300

Glucose 3 gm.

This material was tubed in 5 ml. amounts and  
inspissated at 85° C for 1 hour on 3 successive days.

MacConkey's Agar (Difco)

Peptone 17.0 gm.

Proteose peptone 3.0

Lactose 10.0

Bile-salts (No. 3) 1.5

Sodium chloride 5.0

Agar 13.5

Neutral red 0.03

Crystal violet 0.001

Fifty grams of this material was dissolved in one  
liter of distilled water and autoclaved.



Medium I

Tryptophane Assay Medium (Difco) 11.25 gm.

DL Tryptophane (NBC) 0.11

Distilled water 300.0 ml.

Autoclaved at 121.5° C at 15 lbs. for 15 minutes.

Medium II

L Arginine monohydrochloride 0.22 gm.

L Histidine monohydrochloride 0.14

DL Lysine 0.33

DL Aspartic acid 0.22

DL Serine 0.28

L Glutamic acid 1.21

Beta-alanine 0.11

DL Tryptophane 0.11

DL Valine 0.44

L Leucine 0.28

DL Isoleucine 0.28

Glucose 12.0

K<sub>2</sub>HPO<sub>4</sub> 0.3

KH<sub>2</sub>PO<sub>4</sub> 0.3

MgSO<sub>4</sub> 0.012

NaCl 0.006

FeSO<sub>4</sub> 0.006

MnSO<sub>4</sub> 0.006

Sodium acetate 6.0

Distilled water 300.0 ml.



Medium II (cont'd.)

The pH was 7.4. This medium was tubed in 5 ml. amounts then autoclaved at 15 lbs. for 15 minutes.

Medium III

DL Threonine*	0.025 gm.
DL Serine	0.025
Beta-alanine	0.06
DL Valine	0.075
L Leucine	0.085
Glycyl glycine	0.025
DL Proline	0.035
Hydroxyproline	0.04
DL Aspartic acid	0.09
L Glutamic acid	0.045
DL Phenylalanine	0.04
DL Tyrosine	0.025
L Arginine	0.025
L Histidine monohydrochloride	0.025
DL Lysine	0.045
Glucose	10.0
NaH <sub>2</sub> PO <sub>4</sub>	0.03
Na <sub>2</sub> HPO <sub>4</sub>	0.03
MgSO <sub>4</sub>	0.012
NaCl	0.006
Sodium acetate	0.6
Distilled water	300.0 ml.

continued on page 123



Medium III (cont'd.)

The pH was 7.4. This medium was tubed in 3 ml. amounts and autoclaved at 15 lbs. for 15 minutes. To each tube 2 ml. of the following medium was added aseptically. after it was passed through a Seitz filter.

Thiamine hydrochloride (BDH)	0.0016 gm.
Nicotinamide (Eli Lilly and Co.)	0.0610
DL Methionine	1.4921
DL Cystine	0.4008
DL Tryptophane	0.10211
Fe(NH <sub>4</sub> ) <sub>2</sub> (SO <sub>4</sub> ) <sub>2</sub>	0.006
NaNO <sub>3</sub>	0.006

\*All of the amino acids used were manufactured by the Nutritional Biochemicals Corporation, Cleveland.

Nitrate Medium

Tryptone (Difco)	20.0 gm.
Na <sub>2</sub> HPO <sub>4</sub>	2.0
KNO <sub>3</sub>	1.0
Glucose	1.0
Agar (Difco)	1.0
Distilled water	1.0 l.

Nitrate Solution A

Sulphanilic acid	8.0 gm.
Acetic acid 5N	1.0 l.



Nitrate Solution B

Alpha-naphthylamine 5.0 gm.

Acetic acid 5N 1.0 l.

A few drops of each of these reagents (Nitrate Solutions A and B) were run into the broth culture to be tested. A distinct pink color indicated the presence of nitrite.

Peptone Broth

Peptone (Difco) 15.0 gm.

NaCl 7.5

Distilled water 150.0 ml.

Physiological Saline

NaCl 9 gm.

Distilled water 1 l.

Ringer's Solution

NaCl 0.65 gm.

CaCl<sub>2</sub> 0.012

KCl 0.014

NaHCO<sub>3</sub> 0.02

Distilled water 100.0 ml.

Soft Agar

Nutrient broth 1 l.

Agar (Difco) 3 gm.



Special Anaerobic Transport Medium

A quantity of 200 ml. was found convenient to prepare at one time; 190 ml. of previously autoclaved 0.3% agar (Difco) in distilled water was melted and 0.2 ml. of thioglycollic acid (BDH) added. Sufficient N/l HCl was incorporated to bring the mixture to approximately pH 7.2, and then 10 ml. of 20% sodium glycerophosphate in distilled water was added, along with 2 ml. of a similar 1% solution of calcium chloride. The medium was mixed thoroughly, and while still hot was titrated to pH 7.4 with N/l HCl. Then 0.4 ml. of methylene blue (0.1% in water) was added to give a final concentration of 1 in 500,000, and the medium placed in flowing steam for a few minutes before distribution in 1/4 oz. screw cap "bijou" bottles, about 7 ml. in each. The bottles with the medium were sterilized for one hour in flowing steam.

Sugar Broths

Lactose, Glucose, Sucrose, Mannite, and Maltose

1% sugar in nutrient broth

1% Andrades indicator

Lactose and glucose were run without pressure for one hour on each of three consecutive days in the autoclave. Sucrose, mannite, and maltose were autoclaved at 121.5° C for 15 minutes at 15 lbs. pressure.



Tyrosine Medium

Nutrient agar	1 l.
Tyrosine	5 gm.

Autoclave at 15 lbs. pressure for 15 minutes

Urea Medium

Peptone (Difco)	1.0 gm.
NaCl	0.5
Monopotassium phosphate	2.0
Phenol red	0.012
Agar (Difco)	20.0
Distilled water	1.0 l.

Autoclave at 15 lbs. pressure for 20 minutes.

Add 0.1% glucose and 2% urea — the glucose solution autoclaved at 15 lbs. for 20 minutes and the urea solution passed through a Seitz filter.

















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